Neural and Behavioural Consequences of Chronic Inflammation following Spinal Cord Injury

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Abstract

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This thesis investigated the influence of chronic inflammation on several neural/behavioural disorders following spinal cord injury (SCI) including depression, cognitive impairment, neuropathic pain, and somatic nerve deficits.

Ample evidence exists to suggest that the immune system communicates with, and influences the nervous system both centrally and peripherally. Pro-inflammatory cytokines have been shown to influence the nervous system directly by altering ion channel kinetics, as well as indirectly by altering enzyme function thereby resulting in changes in critical neuroactive compounds. Proinflammatory mediators have been shown to up-regulate the enzyme indoleamine 2,3 dioxygenase (IDO) resulting in the accelerated degredation of serotonin precursor tryptophan (TRP) and increased production of TRP metabolites such as kynurenine (KYN). They have also been shown to upregulate the enzyme cyclooxygenase (COX) resulting in the increased production of pain inducing eicosanoids such as prostaglandin E2 (PGE2). Immune dysfunction in the form of chronic inflammation may therefore contribute to the severity of behavioural disorders such as depression and cognitive impairment, as well as neural disorders such neuropathic pain and somatic nerve deficits. SCI is typically associated with not only a state of chronic inflammation but also a drastically higher prevalence of each of the aforementioned neural and behavioural disorders. This makes SCI an ideal population to study the interaction between the immune and nervous systems, and assess the potential

efficacy of novel treatment strategies which target the immune system for the management of such disorders.

A 3-month anti-inflammatory diet was utilized as a treatment intervention for the purpose of reducing chronically elevated levels of pro-inflammatory mediators. This intervention allowed for the assessment of each of the outcome variables of interest at baseline (under an elevated inflammatory status) as well as at 1-month and 3-months during the intervention (under a reduced inflammatory state). Changes in inflammation were assessed by the quantification of serum pro (IL-1β, IL-2, IL-6, IFN-У, TNF-α, CRP) and anti-inflammatory (IL-4, IL-10, IL-1RA) cytokines. Cytokine-induced alterations in enzyme function and corresponding changes in neuroactive compounds were assessed by tryptophan (TRP), the competing amino acids phenylalanine (PHE), tyrosine (TYR), leucine (Leu), isoleucine (Ile), and valine (Val), the tryptophan metabolite kynurenine (KYN), and the pain-inducing eicosanoids prostaglandin E2 (PGE2) and leukotriene B4 (LTB4). In addition to such molecular indices, actual changes in each of the outcome variables of interest were assessed. Levels of depression were assessed by questionnaire via the Center for Epidemiological Studies Depression Scale (CES-D). Cognitive function (in the form of verbal learning) and memory was assessed via the California Verbal Learning Test (CVLT). Neuropathic pain was assessed via the Neuropathic Pain Questionnaire (NPQ). Somatic nerve function was assessed by EMG, including the assessment of nerve conduction velocity and signal amplitude in both motor and sensory nerves.

The intervention significantly reduced serum concentrations of pro-inflammatory mediators in the treatment group (n=12) by 28%, while no significant change was found

in the control group (n=8). Among other changes in amino acids, the most notable was that the change in the KYN/TRP ratio (an indicator of IDO activity) and the TRP/LNAA ratio (an indicator of TRP availability for serotonin synthesis) was significantly different between groups. The treatment group showed a significant reduction in scores of depression, as well as a significant reduction in sensory neuropathic pain scores. No significant changes were observed in regards to somatic nerve conduction and most indices of cognitive function (with the exception of the ability to avoid incorrect responses on the CVLT).

These results may suggest a substantial role for chronic inflammation in depression and neuropathic pain following SCI and provide a potential alternative treatment strategy for the management of such intractable disorders.

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List of Abbreviations

3-HK	3-Hydroxykynurenine
5-HT	Serotonin
AIS	ASIA (American Spinal Injury Association) Impairment Scale
BBB	Blood brain barrier
BCAA	Branch Chain Amino Acid
CES-D	Center for epidemiological studies depression scale
CMAP	Compound motor action potential
COX	Cyclooxygenase
CRP	C reactive protein
CVLT	California verbal learning test
IDO	Indoleamine 2,3-dioxygenase
IFN-y	Interferon gamma
IL-10	Interleukin-10
IL-1RA	Interleukin-1 receptor antagonist
IL-1β	Interleukin-1 beta
IL-2	Interleukin-2
IL-4	Interleukin-4
IL-6	Interleukin-6
KAT	Kynurenine aminotransferase
KMO	Kynurenine monooxygenase
KYN	Kynurenine
KYNA	Kynurenic Acid
LNAA	Large Neutral Amino Acid
LOX	Lipoxygenase
LTB4	Leukotriene B4
NCV	Nerve conduction velocity
NMDA	N-methyl-D-aspartate
NPQ	Neuropathic pain questionnaire
NSAID	Non-steroidal anti-inflammatory drug
PGE2	Prostaglandin E2

PHE	Phenylalanine
QUIN	Quinolinic Acid
SCI	Spinal cord injury
SERT	Serotonin transporter
SNAP	Sensory nerve action potential
SSRI	Selective serotonin reuptake inhibitor
TDO	Tryptophan 2,3-dioxygenase
TNF- α	Tumour necrosis factor alpha
TPH	Tryptophan Hydroxylase
TRP	Tryptophan
TYR	Tyrosine
α7AChR	alpha 7 nicotinic acetylcholine receptor

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Chapter 1

Introduction

Traditionally, the human body has been studied as a number of individual, isolated systems which carry out unique and independent functions. However, there is now well-established and growing evidence that the body functions as one entity whereby each 'system' acts synergistically with the ability to communicate and influence one-another. This has important implications regarding the way in which diseases are characterized and treated. Although a disorder or disease may be characterized as belonging to one system, in reality, this is rarely (if ever) the case. Complex, bidirectional communicatory pathways exist between systems of the body making it possible for various systems to contribute to a disorder. An understanding of this phenomenon is of critical importance as it may allow for a more holistic understanding of various diseases and result in more effective treatment strategies.

Chronic inflammation plays an increasingly appreciated role in the pathogenesis of a number of neurological and behavioral disorders.[1–3] Communicatory pathways between systems allows for immune dysfunction to contribute to both neural and endocrine impairment via a number of inflammatory mechanisms. Proinflammatory mediators possess the ability to directly influence the nervous system by acting on vagal afferents[4], or by crossing the blood brain barrier (BBB) either through leaky sites at the circumventricular organs[5] (brain regions which lack BBB), or via specialized active transporters.[6] Proinflammatory cytokines have also been shown to influence hormone secretion by acting directly on receptors within the hypothalamic-pituitary-adrenal (HPA) axis.[7] Alternatively, a number of cytokines have been shown to indirectly influence neural and endocrine disorders by altering the regulation of enzymes. This may result in a shift in key metabolic pathways resulting in an imbalance in critical neuroactive

compounds. In addition to impacting neural and behavioural disorders, chronic inflammation contributes to the pathogenesis of a number of metabolic disorders, and these disorders in turn, have been shown to contribute to the elevated inflammatory state, creating a viscous cycle.[8–12] The high prevalence of metabolic disorders is among the many factors which contribute to the chronic inflammatory status commonly observed following spinal cord injury (SCI).

Immune impairment and chronic inflammation have been commonly demonstrated following SCI by elevated concentrations of proinflammatory cytokines and autoantibodies which are apparent in individuals who are symptomatic or asymptomatic for secondary health complications.[13–17] As such, this population is often found to be in a perpetual low-grade inflammatory state which is elevated to an even further extent when other health complications and associated disorders are present. Due to the complex bidirectional communication between the immune and neuroendocrine systems, damage to the spinal cord often results in the widespread dysfunction in other systems.[18] Both the endocrine and immune systems may be affected due to respective dysregulation of the hypothalamic-pituitary-adrenal (HPA) axis and a loss of direct sympathetic innervation of lymphoid organs. Despite the elevated inflammatory state, SCI is typically associated with a state of immunosuppression and a heightened susceptibility to infection.[18–21] Suppressed function of natural killer (NK) cells, neutrophils, macrophages, and lymphocytes have each been documented following SCI.[18,22,23] The loss of motor and sensory function also contribute to this populations' greater susceptibility to a number of acute infections including urinary tract infection

(UTI) and pressure ulcers as well as metabolic disorders associated with a more sedentary lifestyle such as obesity, atherosclerosis, and type 2 diabetes.[24–26]

There is evidence to suggest disorders such as depression, cognitive impairment and neuropathic pain share a common inflammatory etiology stemming from a cytokine-induced imbalance of neuroactive compounds. Individuals diagnosed with major depression have been consistently reported to demonstrate elevated levels of proinflammatory cytokines,[27,28] while those with neuropathic pain have been shown to have altered levels of inflammatory mediators with elevations in the proinflammatory cytokines TNF, IL-6, and IL-2 along with reductions in anti-inflammatory cytokines such as IL-10 and IL-4.[13,29] Chronic inflammation has also been suggested to play a role in cognitive deficits via several mechanisms.[3] As each of these disorders have been shown to be far more prevalent following SCI[30–33] (a condition typically associated with an elevated inflammatory status), inflammatory mediators may contribute to the severity of such disorders in this population. It has also been suggested that cytokines may have a direct influence on nerves causing alterations in ion channel kinetics when at elevated concentrations.[34] As such, cytokines and other inflammatory mediators may have the potential to cause somatic or autonomic nerve deficits.

An understanding of how the immune and neuroendocrine systems interact may provide a unique opportunity to treat neural and behavioral disorders by targeting aspects of immune impairment. The reestablishment of immune homeostasis may not only help to improve the body's ability to fight infection and avoid unnecessary consumption of resources, but may also induce beneficial alterations in enzyme regulation, protein balance, hormone levels, and neural function. Currently, the majority of treatment

strategies for conditions such as major depression and pain utilize drugs which target "downstream" enzymes and receptors. As such, these treatments provide temporary relief from symptoms but do not treat the core inflammatory basis of these disorders. Natural anti-inflammatory interventions including a diet consisting of foods and supplements with anti-inflammatory properties may be an effective option for treating inflammation in this population. As this treatment strategy will target the inflammatory basis of many disorders it would be expected to result in a reduction in pro-inflammatory mediators thereby leading to more sustainable long-term immune improvements. Despite this, little research has focused on the use of anti-inflammatory foods and supplements for the treatment of chronic inflammatory conditions and effects specific to those with SCI. Therefore, the use of anti-inflammatory foods and supplements as an intervention strategy and its potential influence on inflammation, depression, cognitive impairment, neuropathic pain, and somatic nerve function in individuals with SCI is the focus of this thesis.

Chapter 2

Literature Review

Background and Epidemiology of Spinal Cord Injury

Epidemiology and Demographics

The incidence rate of SCI in North America has been on the rise over the past thirty years and currently stands at 35 and 40 per million population per year in Canada and the United states respectively.[35] Of these individuals, an estimated 55% have incomplete SCI, meaning some degree of motor and/or sensory capabilities are maintained below the lesion. In regards to the level of the SCI, those to the cervical region are the most common, accounting for approximately 51% of all cases. Lower level SCI such as those affecting the thoracic and lumbar region are less common accounting for 21% and 28% respectively.[36]

The mean age of individuals living with SCI has traditionally been quite young at approximately 32 years of age. However, over the past thirty years an increase in the number of older adults living with SCI has become evident, due in part to advancements in treatment and care. In 2001, Sekhon and Fehlings reported an increase in SCI occurring in older adults from 4.7% in the 1970's to 10% in 2001.[37] The mean age in North America is now approximated at 42.2 years of age.[38] Despite the shift in the mean age of individuals living with SCI, when concerning the age of onset, young males are still deemed to be at the highest risk and outnumber females by a ratio of 3:1.[39]

Etiology

Etiological data suggests that the primary cause of SCI in North America is motor vehicle accidents, accounting for approximately 50% of all cases.[40] This includes injuries sustained to the driver or passenger of a vehicle as well as to those hit by vehicles. Falls are regarded as the second most common cause accounting for approximately 24% of all

cases. Although SCI caused by falls are only half as common as motor vehicle accidents, they are the only cause which has steadily increased over the past 30 years. Sports related injuries have shown a decline over the past 3 decades and are now responsible for only 9% of injuries. Lastly, injuries caused by violence showed a steady increase, until peaking in the mid 1990's, however, they have since declined to an estimated 11.2%.

Based on an estimated prevalence of 755 per million population, approximately 250 000 people are currently living with a SCI in North America.[40] This creates a need for further research and new treatment methods to help alleviate the physiological, psychological, and socioeconomic impact placed on individuals with SCI. As the location and severity of a SCI are highly variable from one individual to the next, no two injuries are ever exactly the same. Therefore, the development of treatment methods which can be individualized for each patient's unique pathology is of extreme importance.

Anatomy of the Spinal Cord

The spinal cord provides a pivotal conduit between the brain and body in which all motor and sensory information travels. Ascending tracts carry sensory information such as touch, temperature, pain, and joint position to the brain whereas descending tracts carry motor signals to muscle groups throughout the body to initiate movement. The spinal cord is composed of outer spinal tracts known as white matter, and an inner segment comprised of neuronal cell bodies and interneurons known as grey matter. Contained within the protective sheath of the vertebral column, and surrounded by a cushion of cerebral spinal fluid, the delicate tissue of the spinal cord relays critical signals to the limbs, trunk, and organs. These signals exit and enter the spinal cord via small spinal roots which branch out through spaces known as foramina within the vertebral

column. These roots then travel to their target tissue and innervate them by means of neurotransmitter release.

Innervation of the Spinal Cord

The spinal cord is categorized into five regions, each of which is further divided into a number of segments. Each spinal segment is responsible for innervating a unique muscle group or organ as well as receiving sensory information from a particular region of the body. Groups of muscles receiving innervation from a specific spinal segment are known as myotomes. A single spinal segment typically innervates more than one myotome, just as most muscle groups are innervated by more than one spinal segment. This overlap may have important functional implications concerning damage to the spinal cord. If a particular region of the spinal cord is to become damaged, resulting in loss of innervation to the myotome, partial innervations may still be possible from a more rostral segment. This allows for the partial preservation of motor abilities to that muscle group. Specific areas of the skin responsible for relaying sensory information to particular areas of the spinal cord are termed dermatomes. Unlike myotomes however, dermatomes generally provide information to only one spinal segment. Due to the complex overlaying innervations in addition to varying levels, severities, and the specific location of damage to the motor neuron, spinal cord injuries (SCI) are highly variable and often differ greatly from one individual to the next.

Injury Level

The level of spinal tissue damage will vary depending on the nature of the accident or disease and may affect one spinal segment or span over several segments. As each segment of the spinal cord is responsible for innervating a particular myotome and

receiving information from a particular dermatome, the level of the injury will dictate the diagnosis of either tetraplegia or paraplegia. Tetraplegia refers to the loss or impairment of motor and/or sensory function due to a lesion in the cervical region of the spinal cord. As an injury to the cervical region is a high level injury, innervations to the upper extremities, in addition to the lower extremities, trunk, and pelvic organs will all be affected. Paraplegia refers to the loss or impairment of motor and/sensory function due to a lesion in the thoracic, lumbar, or sacral region. Due to the relatively lower level of the injury, the upper extremities remain unaffected while the degree to which the lower extremities, trunk and pelvic organs are affected vary depending on the specific spinal segment affected.

Severity

A SCI may widely vary in severity or the completeness of the injury. A complete injury is one in which there is no sensory or motor function at some point below the level of the lesion. However, with less severe injuries there is a greater sparing of neural tissue and improved functional outcome. Such injuries are termed incomplete and the potential for the exchange of some sensory and/or motor information remains. The completeness of an injury is determined by the presence or absence of motor and sensory capabilities in the fourth and fifth sacral regions of the spinal cord. If any amount of sensory or motor ability exists, the injury is deemed incomplete. This classification can be further specified by considering the amount of motor and sensory capabilities preserved following SCI. By means of the American Spinal Injury Association (ASIA) impairment scale (AIS) an individual's motor and sensory function can be evaluated based on a 5 tier scale. The scale ranges from AIS A, denoting a complete injury, to AIS E, signifying the individual

has normal motor and sensory capabilities. A grade of B, C, or D, indicates an incomplete injury and specifies the degree to which motor and sensory capabilities are maintained (see appendix for precise definition of each classification).

Dyscomplete SCI

In certain cases, a SCI may be clinically classified as complete (i.e. no sensory or motor function corresponding to the s4-s5 spinal segment) despite the preservation of the structural integrity of a number of spinal tracts (as revealed by MRI). In situations such as these, the spinal cord will have a structural appearance much like that of an incomplete injury and the preservation of partial motor and sensory function below the level of the lesion would be expected. The fact that there is an absence of function below the lesion, (specifically in the 4^{th} and 5^{th} sacral regions) despite a lack of structural damage to all tracts, suggests that a non-structurally based mechanism must be responsible for the apparent conduction deficits. It has been hypothesized that molecules seen at high levels during periods of physiological or psychological stress may be responsible. As SCI is a condition typically characterized by a state of chronic inflammation and immune dysfunction, such immune mediators may contribute to neural deficits in this population.

The Nervous System

Signal Transmission

An individual axon can reach lengths of up to one meter, and as such, the ability to transmit signals rapidly while maintaining signal strength is of extreme importance. Supporting cells of the peripheral nervous system known as Schwann cells ensure the structural integrity of the neuron is maintained, and thereby act to sustain signal strength. By growing around the axon and producing several layers of lipid based protective

covering known as myelin, Schwann cells act to insulate the axon. As lipids are poor conductors of electrical current, this covering provides a strong insulator to help contain travelling signals.

The axon is segmented into regions of both myelinated and non-myelinated areas. Although myelinated areas are extremely important concerning efficient signal transmission, non-myelinated areas are necessary for the re-initiation of action potentials. Signals are typically generated at a region where the axon joins the cell body known as the axon hillock. As the signal travels along the axon it is re-initiated at non-myelinated regions known as Nodes of Ranvier. These regions are densely populated with voltage regulated gates which allow the signal to be boosted back to its original strength. This process occurs at an extremely rapid rate and is termed saltatory conduction due to the way the signal seemingly jumps from one node to the next.[41]

At the site of the axon hillock and each Node of Ranvier, the exchange of sodium and potassium ions causes an alteration in the resting membrane potential. As the action potential generated from the previous node travels to the next, the membrane becomes depolarized, stimulating the opening of a number of sodium channels. This allows an influx of sodium (Na^+) to diffuse into the cell, leading to further depolarization and stimulating the opening of additional Na^+ channels. If this depolarization meets the threshold of approximately -55mv, the triggering of a new action potential will result. Upon reaching threshold, Na^+ channels close, preventing further depolarization, and potassium (K^+) channels open, allowing an efflux of K^+ ions to diffuse out of the cell, causing repolarization and the return of resting membrane potential.[42] These action potentials are non-graded, meaning that regardless of the strength of the stimulus applied,

as long as the threshold is met, a standardized amplitude action potential will always be produced. These action potentials are also non-decremental meaning they maintain their strength as they travel along the axon. This is due to the constant re-initiating of the action potential at each Node of Ranvier. As the action potential arrives at the next node in the sequence the signal will be just strong enough to cause opening of the voltage gated channels and initiate a new action potential of equal strength to that of the previous node. Electrical signaling such as this is an effective communicatory pathway but can be performed only along a single continuous axon. Therefore, when an impulse must transition between two neurons, or from a neuron to a muscle fiber, such as that seen at the site of the neuromuscular junction, a different means of signaling must be utilized. This is accomplished through chemical signaling by means of neurotransmitter release.[43]

Upon reaching the targeted skeletal muscle, the electrical impulse traveling along the pre-synaptic neuron must be converted to a chemical signal, cross the synaptic cleft, and initiate a new electrical impulse on the post-synaptic muscle fibre. This is accomplished through the release of the neurotransmitter acetylcholine (ACh) from vesicles within the pre-synaptic neuron. Upon being released, ACh crosses the synaptic cleft whereby it binds to receptors which open chemically gated channels.[43] This allows Na^{+} ions to flow into the muscle fibre causing depolarization of the resting membrane potential and the production of a new action potential.

The newly generated impulse then travels along specialized tubes known as transverse tubules (T-tubules) which carry the signal deep into the muscle fiber. These T-tubules transport the impulse to an internal membrane system responsible for housing calcium (Ca^{++}) ions known as the sarcoplasmic reticulum (SR). Upon stimulation of the

SR, Ca^{++} ions are released into the cell.[44] This release is a crucial component in the contraction of skeletal muscle, and is required for the functioning of specialized contractile proteins. Contractile proteins including a thick filament composed of myosin and a thin filament composed of actin cause the contraction of skeletal muscle through the formation of crossbridges. Molecules of actin have binding sites for myosin which, through an energy dependent process, allow for contraction by means of a process known as the sliding filament theory. However, until stimulated by the release of Ca^{++} ions, these binding sites remain blocked by a protein known as tropomyosin. The binding of Ca^{++} ions to an associated protein known as troponin causes a conformational change in tropomyosin, revealing the actin binding sites and allowing for crossbridge formation.[44] The myosin heads may then perform a power stroke causing a shortening in sarcomere length, and ultimately a skeletal muscle contraction.

Somatic Nerves

Peripheral nerves consist of bundles of axons which exit and enter the central nervous system from various sites along the spinal cord and brain stem. They are categorized by the direction in which they carry signals and the function which is carried out by those signals. Peripheral nerves of the somatic nervous system are responsible for relaying sensory, afferent signals throughout the body to the CNS as well as motor, efferent signals from the CNS to skeletal muscles for the voluntary contraction of muscles.

Assessment of Somatic Nerves

Both motor and sensory nerves can be assessed via electrical stimulation. Through stimulation of the median nerve and recording of the resulting motor response of the

flexor pollicis brevis, motor nerve conduction velocity as well as the strength of the corresponding motor response may be assessed. Supramaximal stimulation of the nerve results in the recording of a waveform known as a compound muscle action potential (CMAP). This waveform represents the summation of individual motor unit action potentials and can be used to assess the health of motor nerves. Sensory nerves may also be assessed via the recording of sensory nerve action potentials (SNAP). These signals may be recorded in either an orthodromic or antidromic fashion. The median nerve may be assessed orthodromically by stimulating the 4th finger by means of ring electrodes and recording the response over the median nerve at the wrist. Reversing the stimulation and recording sites allow for the recording of an antidromic signal. The SNAP waveform represents the summation of individual impulses traveling through all stimulated sensory nerves and is recorded directly from the sensory nerve. This results in a much smaller amplitude than that of a CMAP. However, through the application of a supramaximal stimulation, SNAPs may also be used to assess nerve conduction velocity and SNAP amplitude.

Autonomic Nerves

Nerves of the autonomic nervous system are responsible for relaying signals for the control of a variety of involuntary processes. Afferent, signals are sent from a number of structures throughout the body such as the blood vessels, heart, or sweat glands and travel to the medulla, pons, and hypothalamus where they do not reach conscious thought. Efferent signals are then relayed to various structures where they elicit involuntary excitatory or inhibitory effects. The autonomic nervous system is further divided into sympathetic and parasympathetic divisions based on these effects. The

sympathetic division is responsible for inducing the “fight or flight” response, causing such responses as increases in heart rate, vasoconstriction of blood vessels, or activation of sweat glands. The parasympathetic division is responsible for inducing the “rest and digest” response, causing such responses as decreases in heart rate, and the promotion of responses necessary for digestion such as salivary gland stimulation and accelerated peristalsis.

Assessment of the Autonomic Nervous System

Heart rate variability (HRV) examines the variation in time between normal heartbeats and can be used to assess the state of cardiac autonomic regulation. More specifically, HRV is used as an indicator of autonomic modulation of the heart. HRV may be assessed in various domains including the time domain, frequency domain, or by means of non-linear methods. Both time and frequency domain indices of HRV are closely related however, frequency domain analysis allows for the differentiation between sympathetic and parasympathetic components. The performance of a spectral analysis via a fast-Fourier transformation allows for signals to be separated into low and high frequency components. Low frequency signals are those found between 0.04-0.15Hz and represent both sympathetic and parasympathetic modulation of the heart. High frequency signals are those found between 0.15-0.4Hz and represent parasympathetic modulation of the heart. From this, the LF:HF ratio may also be used as an estimate of sympathovagal balance. A parasympathetically dominated nervous system at rest results in increased HF power and is considered healthy, whereas sympathetic domination results in a an increased LF:HF ratio and is associated with an increased risk of heart disease.

Susceptibility of the Nervous System to Neuropathy

The above describes neuronal functioning and signal transmission under normal healthy conditions. With the support from glial cells, neurons of the somatic and autonomic nervous system function to send and receive signals throughout all regions of the body. This system is however, extremely sensitive to any disturbances and, in addition to physical trauma, may be influenced by alterations in other systems of the body including the endocrine and immune systems.

Immunoregulatory Function

Cytokine Production

Cytokines are small signaling proteins involved in an array of immunoregulatory responses including hematopoiesis, and inflammation.[45] Historically, they have been viewed solely as molecules produced and released by a variety of immune cells in response to an immune stimulus. They may be produced by cells within the brain and spinal cord such as neurons, astrocytes, and oligodendrocytes, or by a variety of circulating immune cells.[34] More recently, cytokines have been shown to be associated with exercising skeletal muscle. These cytokines termed "myokines" are produced by skeletal muscle and may act to help regulate glucose levels during periods of intensive exercise.[46]

The Immune System

The immune system is responsible for protecting the body against foreign, potentially pathogenic, molecules which would otherwise cause harm through infection or disease. Human immunoregulation is a complex process characterized by two branches of immunity; the innate and adaptive systems.[47] Each system works synergistically on

both systemic and local levels to recognize, attack, and destroy foreign materials. Upon initial attack from a foreign molecule the innate system is activated. The innate system uses non-specific defenses including physical barriers such as the skin and mucous membranes, cells such as phagocytes and natural killer cells, and soluble factors such as complement to prevent infection.[48] If the defenses of the innate system fail, the adaptive system is activated and pathogen specific defenses are used to fight off infection. A variety of lymphocytes are able to recognize antigens by means of surface receptors and produce specific defensive proteins and antibodies to respond.[49]

Innate Immunity

Within the innate immune system, cells known as phagocytes including macrophages, dendritic cells, and neutrophils, act on foreign molecules, engulfing and destroying them through a process known as phagocytosis.[48] Virally infected cells and cancer cells are dealt with in a different manner through a process known as apoptosis in which the cell is stimulated to undergo self-destruction. In this process, specialized cells of the innate immune system known as natural killer cells prevent viral spreading by releasing pore-forming proteins into the infected cell leading to eventual lysis.[50] Natural killer cells, in addition to macrophages, are among the most prolific cytokine producers of the innate immune system and through this production, assist in the recruitment of additional immune cells during an immune response.[50] These cytokines may function in autocrine or paracrine fashions and can cause a variety of responses including the up-regulation or down-regulation of the production of other cytokines, an increase in the number of a particular surface receptor, or the suppression of their own effects through feedback inhibition.[51]

Adaptive Immunity

Immune cells known as lymphocytes, belonging to the adaptive immune system, produce specific responses to invading antigens. The adaptive immune system may be categorized into either humoral or cell mediated immunity. Humoral immunity deals with extracellular pathogens by means of circulating antibodies. When an antigen is present in circulation, phagocytes such as macrophages, monocytes, and dendritic cells, which function as antigen presenting cells (APC's), are able to initiate an immune response.[49] Receptors on the surface of APC's are activated by molecular patterns associated with a pathogen, leading to the increased expression of a protein found on the cell surface known as major histocompatibility complex 2 (MHC-2).[52] This protein is able to accept some of the foreign digested proteins of the antigen after it has been destroyed by the phagocyte. It can then present this matter to either a naïve T-lymphocytes or a memory T-lymphocyte. Naïve T-cells are a subset of T-cells which have never before encountered an antigen. Upon being presented with a new antigen a primary immune response is initiated in which the T-cell undergoes extensive proliferation within the lymph nodes producing further copies of T-cells capable of recognizing the specific antigen.[49] Memory T-cells are those which have previously encountered the antigen, and as such, can produce a faster, stronger secondary immune response following re-exposure.[53] Upon being presented with the antigen, T-helper cells, particularly effector cells, are stimulated to release cytokines. These cytokines cause the proliferation and differentiation of B-cells into memory cells as well as immunoglobulin secreting plasma cells. The antibodies produced by these new plasma cells do not directly destroy antigens but instead bind to specific antigens and assist in their destruction. Upon binding to the antigen, the antibody may act to block the toxic actions of the antigen, or cause the clumping of several

antigens making them more easily phagocytized.[54] In addition, antibodies activate circulating complement which coat antigens increasing the likelihood of phagocytization.[54] Antibodies are an effective, specialized means of destroying circulating pathogens. However, they are unable to enter the cell and act intracellularly.[54] As such, if the pathogen has already entered a cell, different means of immunity are necessary.

Cell Mediated Immunity

Cell mediated immunity utilizes activated T-cells in order to attack and destroy infected host and foreign cells. T-helper cells bind to the surface of macrophages whereby MHC proteins display antigenic determinants.[52] This binding causes the macrophage to release the cytokine interleukin-1 (IL-1) which stimulates the proliferation of additional T-cells. The activated T-cells also release additional cytokines known as interleukin-2 (IL-2) which cause further proliferation of T-cells including T-helper cells and T-cytotoxic (Tc) cells.[49] These Tc cells recognize and attach to cells expressing the same antigen determinants as the original MHC protein. In a process similar to that of natural killer cells, the Tc cell releases perforin into the infected cell causing lysis.[49] The remnants of the destroyed cell are then engulfed and cleared by phagocytes.

The Inflammatory Response

The role cytokines play in cellular communication during both innate and adaptive immune responses is an essential component of the body's defenses. Without the increased recruitment and proliferation of necessary immune cells, the body would be unable to cope with invading pathogens and tissue damage brought on by infection would result. Although healthy individuals are generally able to fight off infection, especially

when confronted by a pathogen dealt with in the past, at times the body does succumb to tissue damage.

Whether due to infection or physical trauma, the inflammatory response is triggered in order to prevent the spreading of any pathogens, and heal damaged tissue. After the onset of trauma, vasodilators such as histamine and bradykinin are released by specific leukocytes known as mast cells and basophils. As a result, vessels are triggered to vasodilate allowing for an increase in blood flow to the site of the injury.[55] This hyperemia causes an increase in the rate of delivery of different immune cells, as well as the removal of wastes and toxins. In the initial stages of the response, clotting proteins such as fibrinogens travel to the injury site and help prevent the spreading of pathogens by forming a sticky mesh in the areas adjacent to the tissue damage.[56] Once the pathogens are contained, phagocytic cells such as macrophages and neutrophils begin to engulf and destroy any foreign molecules. Additional phagocytes are recruited from the circulation by means of a chemical attraction to the cytokines released by local neutrophils through a process known as chemotaxis.[55] As these newly recruited phagocytes travel towards the site of inflammation they are seized by the sticky membranes of local endothelial cells due to the local production of cell adhesion molecules.[57] In addition to increased recruitment, enhanced leukocyte proliferation from red bone marrow is stimulated by the release of cytokines from activated T-cells through a process known as leukopoiesis. Together, this increase in leukocyte proliferation and recruitment aid in the inflammatory response and help return the damaged tissue to a healthy state.

Cytokine Homeostasis

The classification of cytokines is made extremely difficult due to their redundant and pleiotropic properties.[58] This causes a large degree of overlap, making the categorization of cytokines a daunting task. One such method which can be used to distinguish the properties of one cytokine from another is by their overall effect on the inflammatory process. In this sense cytokines may be deemed as either pro-inflammatory or anti-inflammatory. Pro-inflammatory cytokines act to enhance the inflammatory process by promoting leukocyte recruitment and proliferation.[59] In healthy individuals an elevation in pro-inflammatory cytokines will be seen during periods of trauma or infection. These cytokines possess the ability to pass freely through the blood brain barrier and up-regulate their own expression.[60] As the damaged tissue is repaired and bacteria and foreign matter are engulfed and destroyed, a rise in anti-inflammatory cytokines occurs. These anti-inflammatory cytokines act as antagonists and deactivate many of the cytokine-producing immune cells to help return cytokine concentrations to basil levels.[61] This cytokine balance is maintained in healthy individuals but a disruption in cytokine homeostasis has been shown to be related to several conditions including SCI.

Immune Dysfunction and Chronic Inflammation Following SCI

Bidirectional communicatory pathways between the nervous, endocrine, and immune systems create a complex multifaceted etiological basis to immune dysfunction following SCI. Direct insult to the nervous system may induce immune dysfunction via the loss of autonomic innervation of lymphoid organs and corresponding endocrinal dysfunction stemming from HPA axis dysregulation. Damage to the somatic nervous system and the corresponding loss of motor and sensory function may also increase the

risk of developing a number of secondary health complications as well as metabolic disorders associated with a state of inflammation. In addition, a number of related disorders associated with a state of chronic inflammation have been found to be at a substantially higher prevalence following SCI. Together, such factors help explain the chronic inflammatory state and immune impairment typically observed following SCI.

Autonomic Nerve Damage

The immune system is under neuromodulatory control via the direct innervation of primary and secondary lymphoid tissues by autonomic nerve fibers of the sympathetic nervous system (SNS).[22] Preganglionic sympathetic neurons originating from the spinal column synapse in ganglia with peripheral sympathetic neurons. These nerve fibers are responsible for innervating several lymphoid organs and their blood vessels including the spleen, thymus, and lymph nodes, whereby they act to regulate blood flow as well as communicate directly with immune cells.[22] Within these organs, postganglionic noradrenergic fibers release catecholamines, such as norepinephrine (NE), for direct communication with lymphocytes which bear receptors for various neurally active hormones, neurotransmitters, and neuropeptides.[62] The initial release of catecholamines has been shown to induce a rapid, transient increase in blood lymphocytes, however, prolonged exposure induces a number of anti-inflammatory effects including reduced circulating lymphocyte numbers and suppressed NK cell activity.[20] Prolonged exposure to catecholamines also seem to inhibit proinflammatory cytokine production by Th1 cells, while having no effect or even acting to enhance the production of anti-inflammatory cytokines by Th2 cells.[20]

As lymphoid organs such as the spleen and adrenal gland are innervated by sympathetic neurons which originate from regions throughout the thoracolumbar spinal cord, an injury at or above this region may be expected to induce immune suppression.[21] Damage to the cervical spine would interfere with supraspinal control over preganglionic neurons below the injury, whereas damage at the mid-thoracic region would damage preganglionic sympathetic neurons directly.[19] Level dependent impairment in B-cell function has been demonstrated in mice subjected to high (T3) vs mid-thoracic (T9) SCI. It was shown that only after high thoracic SCI (causing disruption of autonomic control of the spleen) was splenic NE elevated and immune function suppressed,[21] whereas mid-thoracic SCI (leaving autonomic control of the spleen intact) has been shown to induce B-cell activation, increase the synthesis of autoantibodies[63] and activate autoreactive T cells.[64] Catecholamines such as noradrenaline have also been shown to be significantly reduced in humans with cervical or high thoracic injuries and have been attributed to a loss of adrenal gland innervation and dysfunction in sympathetic pathways.[65,66] Alternatively, certain aspects of post SCI immune alterations appear to be independent of injury level. Similar alterations in NK cell and T cell cytotoxic activity have been demonstrated in humans with both high and low level injuries,[23,67] as have elevations in viral load following infection in mouse models of high and low thoracic SCI.[19] It may be possible that the level of injury has a greater impact on certain aspects of immunity than others. Additional factors such as autonomic completeness of injury, and time since injury may also explain some of the variability in findings.

Damage to the SNS may also contribute to reduced immune function due to a loss of afferent signaling from the adrenal gland to the hypothalamus. A loss of such afferent

pathways would reduce facilitation of hypothalamic nuclei and may ultimately induce dysfunction in the HPA axis.[18] Lesions on the anterior hypothalamus have been shown to lead to diminished numbers of nucleated spleen cells and thymocytes as well as diminished antibody production, while lesions on the medial hypothalamus have been shown to lead to diminished T and B cell numbers.[18] It may be possible to speculate that damage to sympathetic afferents and corresponding reductions in the facilitation of hypothalamic nuclei could produce similar effects to that observed under conditions of hypothalamic lesions and contribute to the HPA axis dysfunction commonly reported following SCI.

Endocrine Dysfunction

The hypothalamic-pituitary-adrenal (HPA) axis provides an additional immunoregulatory pathway whereby the immune response may be influenced hormonally. Within this pathway the release of corticotropin releasing hormone (CRH) from the hypothalamus induces the release of adrenocorticotropic hormone (ACTH) from the pituitary, which ultimately stimulates the release of glucocorticoids such as cortisol from the adrenal gland. Glucocorticoids induce immunosuppressive effects by acting on leukocytes equipped with receptors for various stress hormones. Once bound, glucocorticoids induce the up-regulation of anti-inflammatory cytokines as well as the suppression of proinflammatory cytokines and other proinflammatory mediators.[68] Glucocorticoids also suppress the maturation, differentiation, and proliferation of a number of immune cells. Due to this critical hormonal influence, an imbalance in the HPA axis in either direction may result in immune dysfunction. Excessive glucocorticoid production may result in immunosuppression and an increased risk of infection, whereas

a non-responsive HPA axis may result in a lack of suppression resulting in an elevated inflammatory state.

Proinflammatory cytokines possess the ability to up-regulate the HPA axis and as such, may contribute to HPA axis over-activation under conditions of chronic inflammation as typically observed following SCI. Such an influence may lead to the excessive production of glucocorticoids from the adrenal gland causing elevated circulating levels and a greater immunosuppressive influence. Increased levels of urine-free cortisol, stimulated by elevated plasma ACTH, have been demonstrated in humans following SCI and have been shown to remain elevated for 3-months post injury.[18,69] This process may induce the hormonally driven suppression of both the innate and adaptive immune systems and explain in part, the increased susceptibility to infection and reduction in wound healing capabilities observed following SCI.[70] Such an influence has been demonstrated within the innate immune system by reductions in NK cell function as well as in the adaptive immune system by reductions in T-cell function which were shown to be inversely related to cortisol levels.[18] The majority of studies demonstrating this up-regulation of the HPA axis and elevated levels of cortisol have however, typically been performed under acute SCI conditions. It may be possible that under chronic conditions, the ongoing stress placed upon the HPA axis could result in adrenal insufficiencies. Several studies performed on individuals with chronic SCI have eluded to such an effect.

Individuals with chronic SCI have been shown to have reduced basal cortisol levels and a reduced cortisol response to exogenous CRH administration.[71] A similar blunted cortisol response has also been demonstrated following intramuscular injection of

ACTH.[72] Interestingly, in a similar study performed under acute SCI conditions, ACTH injection was shown to produce a normal elevation in plasma hydroxycorticosteroid levels.[73] These results may further support the theory that SCI results in adrenal insufficiencies which take time to develop and are not apparent until the chronic stages. Such effects have been suggested to be the result of mild adrenocortical atrophy and/or a reduction in the sensitivity of corresponding adrenal receptors.[71] Of note, an increased adrenal volume within chronic SCI has been demonstrated in individuals with impaired adrenal reserves, suggesting a reduction in adrenal receptor sensitivity may be the more likely cause.[74] However, due to a limited sample size, further studies are required to make any definitive conclusions regarding the true cause of adrenal insufficiency following SCI. Regardless of the cause, if the adrenal gland does not sufficiently respond to ACTH released by the pituitary, adrenal insufficiencies may result, causing reduced levels of circulating glucocorticoids and a lack of immunosuppression, resulting in an elevated inflammatory state.[68]

Therefore, whether due to an elevation in glucocorticoid production caused by overstimulation of the HPA axis, or an eventual reduction in glucocorticoid production caused by desensitization of the ACTH receptors on the adrenal gland, immune dysfunction may result. This may explain in part, an endocrine role in chronic inflammation following SCI.

Somatic Nerve Damage

A loss of motor and/or sensory function following SCI places individuals at a heightened risk for the development of a variety of acute infections. Secondary health complications such as urinary tract infections (UTI) and pressure ulcers are common

occurrences in this population, resulting in frequent spikes in inflammatory mediators. A recent study by Street et al. (2013), followed a group of 171 patients receiving acute care for traumatic spinal cord injury and found 77% experienced an adverse event during their stay including UTI's, pneumonia, neuropathic pain, pressure ulcers, and delirium. Of these complications, UTI's were found to be the most common occurrence.[75]

A loss of voluntary motor control of the bladder and/or the ability to sense when the bladder is full, may result in insufficient bladder voidance. This results in the accumulation of stagnant urine within the bladder, allowing for an increase in bacterial growth and a resulting risk of infection.[14] UTI's have been consistently shown to be the most frequent cause of re-hospitalization in the SCI population, accounting for approximately 24-54% of cases.[76] This indicates that UTI's are a common occurrence, not only during the acute stages of SCI, but also long term as well.

Both paralysis and atrophy of skeletal muscle, in combination with a loss of functioning dermatomes, also increases the risk of tissue damage and infection via pressure ulcer development. An inability to shift one's body weight and/or sense the pain and pressure associated with remaining in the same seated or supine position for an extended period of time, may lead to the occlusion of blood flow to an area of tissue. As such, various areas of the body, particularly the gluteal and sacral regions, may be at risk for oxygen deprivation and necrosis.[14] Individuals with SCI are at a particularly high risk for pressure ulcer development with approximately 40% of individuals developing a pressure sore within any three year period.[76]

Acute secondary health complications such as these result in an acute inflammatory response characterized by a spike in proinflammatory mediators.[77,78] The frequent

occurrence of such complications paired with slower healing times makes this population prone to frequent and prolonged elevations in inflammatory mediators, further compounding the already elevated basal inflammatory state.

Metabolic Disorders

Following SCI, the loss of motor function typically leads to inactivity and increases the likelihood of living a sedentary lifestyle. Further, the additional physical, psychological and biochemical demands placed on the body makes proper diet even more crucial to meet the body's elevated nutrient requirements. Unfortunately, a lack of awareness in this area combined with the cost and effort involved in maintaining a healthy diet places this population at high risk for nutrient deficiencies. Together, this places the SCI population at a higher risk for the development of metabolic disorders such as obesity, type 2 diabetes and atherosclerosis;[24–26] each of which are independently associated with a chronic inflammatory status.[8,24]

Obesity is now classified as an inflammatory disorder due to the discovery that adipose tissue acts as an endocrine organ, possessing the ability to secrete proinflammatory mediators, termed adipokines, such as TNF-α, IL-1, and IL-6.[79] As such, an abundance of adipose tissue, as seen in obesity, may result in the overproduction of proinflammatory mediators and contribute to a low-grade inflammatory state. Adipose tissue is also known to secrete adiponectin, a protein with a critical role in fatty acid oxidation and glucose regulation.[80] Elevated concentrations of proinflammatory cytokines have however, been shown to blunt the release of adiponectin thereby limiting its ability to induce fatty acid oxidation and aid in glucose regulation by means of enhanced insulin sensitivity. This decrease in fatty acid oxidation further contributes to

the over-abundance of adipose tissue, which in turn leads to a greater secretion of proinflammatory cytokines, creating a vicious inflammatory cycle. A reduction in plasma concentrations of adiponectin has been reported in individuals with obesity, as well as in those with type 2 diabetes, and cardiovascular disease.[81]

Closely related to this obesity-induced inflammatory state, is a state of insulin resistance associated with the development of type 2 diabetes. Several cross-sectional studies using non-diabetic subjects have demonstrated that those with impaired glucose tolerance displayed elevated concentrations of proinflammatory cytokines such as C-reactive protein (CRP), sialic acid, TNF- α, and IL-6. Additionally, each were shown to be positively correlated with insulin resistance.[10,11,82] In addition to adiponectin related mechanisms, a number of proinflammatory cytokines including TNF-α, IL-1, and IL-6 have each been suggested to play a role in insulin resistance via the down-regulation of GLUT 4 gene transcription and translocation, as well as the inhibition of insulin receptors by mechanisms related to the up-regulation of the suppressors of cytokine signaling (SOCS) proteins.[83,84] Insulin resistance may in turn, result in elevated blood glucose concentrations placing excess strain on the kidneys and ultimately resulting in tissue damage and further inflammation.

Atherosclerotic plaques associated with cardiovascular disease are also influenced by, and further induce, elevations in proinflammatory mediators. A variety of proinflammatory mediators have the ability to induce vasoconstriction, thereby increasing shear force on vessels and the likelihood of endothelial damage. Additionally, proinflammatory mediators act to increase chemotaxis and the production of adhesion molecules thereby increasing leukocyte infiltration and platelet aggregation at the site of

endothelial damage.[12] Together, these mechanism act to increase the risk of plaque formation in the form of an atherosclerotic plaque. Such plaques are treated similarly to that of any other form of tissue damage with the elicitation of an inflammatory response. However, in the case of an atherosclerotic plaque, the inflammatory response is unable to repair the damaged tissue. Instead, migrating immune cells become trapped within the plaque and release inflammatory mediators contributing to an even greater inflammatory response and state of low-grade inflammation.[12]

For these reasons, among others, the highly sedentary lifestyle often adopted following SCI, in combination with a lack of necessary dietary alterations may contribute to the chronic low-grade inflammatory response.

Mononuclear Cells

Following SCI, changes in immune-regulation have been shown to coincide with chronically elevated levels of circulating proinflammatory cytokines. This elevation is apparent in both the acute and chronic stages of SCI and has been shown whether or not the individual is symptomatic for secondary health complications.[13] There is evidence to suggest that this occurs due to a bias in the immune system towards a particular subset of T-cells. T-cells can be categorized into either effector T-cells or regulatory T-cells. Although overall levels stay relatively similar, after SCI there is a shift in the predominant T-cell type whereby a greater proportion of effector T-cells is evident.[13] Regulatory T-cells are crucial in the maintenance of immunological tolerance and produce immunosuppressive effects through the production of anti-inflammatory cytokines.[13] Effector T-cells differentiate into one of two helper T-cell subsets including T-1 helper (Th1) cells or T-2 helper (Th2) cells. Th1 cells are responsible for the

promotion of cell mediated immune responses and predominantly produce pro-inflammatory cytokines such as, interleukin-2 (IL-2), interferon-gamma (IFN-y), and tumour necrosis factor beta (TNF-β). Th2 cells promote humoral immune responses and predominantly produce anti-inflammatory cytokines such as, interluekin-4 (IL-4) and interluekin-10 (IL-10).[45] A shift towards a Th1 cell dominated immune system following SCI would lead to a greater proportion of pro-inflammatory cytokines. This dominance may be due to several factors including antigen quantity, and the initial APC interaction with T-cells.[85] For example, APC's known as dendritic cells will induce the development of Th1 cells when in the presence of high antigen levels. As individuals with SCI are prone to secondary health complications, the resulting pathogen-derived products may influence the T-helper cell response. This shift to a greater proportion of pro-inflammatory cytokines would lead to an up-regulated immune response and potentially a state of chronic inflammation.

Related Disorders

A number of disorders including depression and neuropathic pain are associated with a state of chronic inflammation. Individuals diagnosed with major depression have been consistently reported to demonstrate elevated levels of proinflammatory cytokines,[27,28] and those with neuropathic pain have been shown to have altered levels of inflammatory mediators with elevations in the proinflammatory cytokines TNF, IL-6, and IL-2 along with reductions in anti-inflammatory cytokines such as IL-10 and IL-4.[13,29] As these disorders have a highly elevated prevalence following SCI, they too may contribute to the elevated inflammatory status observed in this population. The prevalence of depression following SCI is estimated to be 5 times greater in comparison to the general

population with rates of approximately 20-40%.[30,31] Neuropathic pain is also far more prevalent, affecting an estimated 29-75% of the SCI population.[32] Although the inflammatory mechanisms behind these disorders are not fully understood, the ability of proinflammatory cytokines to act as neuromodulators allows them to contribute to these disorders via both direct and indirect influences.[5]

Cytokines have the ability to directly influence the central nervous system and play a role in a variety of responses including behaviour modification by accessing the brain through leaky sites of the blood brain barrier (BBB) via the circumventricular organs[5], or utilizing specific active transporters to cross the BBB.[6] Alternatively, cytokines can communicate by acting on the vagal afferents thereby avoiding the need to cross the BBB.[4] Proinflammatory mediators have also been suggested to directly influence nociceptors, by reducing ion channel activation thresholds leading to peripheral sensitization.[86]

Proinflammatory cytokines also possess the ability to indirectly influence neuronal function via their ability to alter the regulation of key enzymes. The chronically elevated levels of proinflammatory cytokines often reported following SCI, may therefore result in chronically up-regulated enzyme activity leading to potential protein imbalances related to both depression and neuropathic pain. For example, a number of proinflammatory cytokines have been shown to up-regulate the enzyme indoleamine 2,3-dioxygenase of the kynurenine pathway. As this enzyme is responsible for the degradation of tryptophan, a critical precursor in the synthesis of serotonin, its chronic up-regulation may result in serotonin deficiencies and related depressive symptoms.[87] Proinflammatory cytokines have also been shown to up-regulate the enzyme

cyclooxygenase (COX). This enzyme is responsible for the production of potent, proinflammatory eicosanoids with established pain inducing properties such as prostaglandin 2 (PGE2).[88] An up-regulation of COX may therefore result in greater and far more frequent neuropathic pain.

It is debated whether an elevated inflammatory status is the product of, or rather the cause of such disorders. However, as both psychological stress and neurological damage would be expected to result in an elevation in proinflammatory mediators, it is likely a combination of effects.

The Inflammatory Etiology of Neural Disorders

The Inflammatory Etiology of Depression and Cognitive Impairment

Both depression and cognitive impairment may share a closely linked inflammatory etiology stemming from a cytokine-induced imbalance in the kynurenine pathway. As this pathway provides the primary route for tryptophan (TRP) degradation, it plays a major role, not only in the maintenance of serotonin (5-HT) synthesis, but also in the critical balance between neurotoxic and neuroprotective metabolites. As such, a state of chronic inflammation, as is commonly reported in cases of depression and severe cognitive deficits, may contribute to the pathogenesis of each of these disorders.[27,28,89,90]

The Kynurenine Pathway

The kynurenine (KYN) pathway involves a cascade of enzymatic steps responsible for the degradation of tryptophan (TRP) into a number of metabolites, known as kynurenines. This pathway is responsible for approximately 95% of whole body TRP metabolism, and is of critical importance concerning the maintenance of several key amino acids with neuromodulatory roles.[91,92] As TRP is the precursor for serotonin (5-

HT) synthesis within the brain, the maintenance of sufficient levels has important implications concerning depression. Additionally, several TRP metabolites such quinolinic acid (QUIN) and kynurenic acid (KYNA) have roles in symptoms of both depression and cognitive impairment, further stressing the importance of a strictly controlled rate of TRP metabolism.

The degradation of TRP is controlled by two rate limiting enzymes known as indoleamine 2,3-dioxygenase (IDO), and tryptophan 2,3-dioxygenase (TDO).[87,93] Together, under the regulation of cytokines, steroids, and growth factors, these enzymes control the conversion of TRP into the first metabolite of the kynurenine pathway; kynurenine (KYN). This metabolite is then further metabolized along one of two distinct branches of this enzymatic cascade including the kynurenine-kynurenic acid (KYN-KYNA) branch and the kynurenine-nicotinamide adenine dinucleotide (KYN-NAD) branch.[87] Within the former branch, KYN is further converted to the metabolite kynurenic acid (KYNA) via the enzyme kynurenine aminotransferase (KAT). The latter branch utilizes the enzyme kynurenine 3-monooxygenase (KMO) to convert KYN to the metabolite 3-hydroxykynurenine (3-HK) and further in the cascade, quinolinic acid (QUIN).

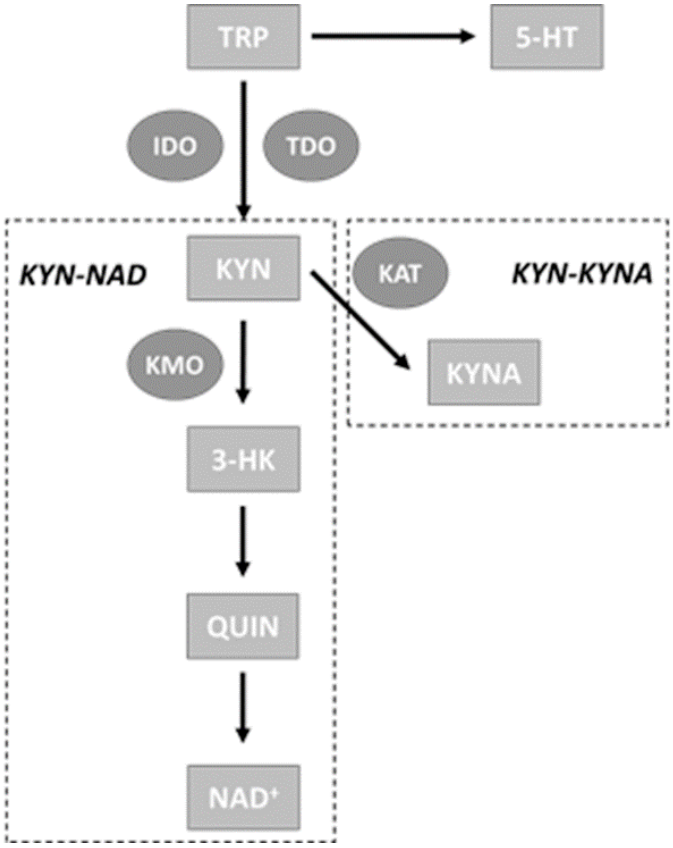

Figure 1: Simplified depiction of tryptophan breakdown in the kynurenine pathway

Tryptophan (TRP) that is not transported across the blood brain barrier (BBB) for the synthesis of serotonin (5-HT) is degraded into kynurenine (KYN) by the enzymes indoleamine 2,3-dioxygenase (IDO) and tryptophan 2,3-dioxygenase (TDO). After this point, KYN is further degraded along one of two distinct branches; either the kynurenine-nicotinamide adenine dinucleotide (KYN-NAD) branch, or the kyneine-kynurenic acid (KYN-KYNA) branch. Within the KYN-NAD branch, KYN is acted on by the enzyme kynurenine 3-monooxygenase (KMO) whereby it is converted to 3-hydroxykynurenine (3-HK) and later quinolinic acid (QUIN) via a spontaneous reaction (and ultimately NAD+). Within the KYN-KYNA branch, KYN is acted on by the enzyme kynurenine aminotransferase (KAT) whereby it is converted to kynurenic acid (KYNA).

The kynurenine pathway has both a peripheral and central component, and as they are not completely autonomous, the central component is heavily influenced by that of the periphery. The rate limiting enzyme TDO has been shown to be highly expressed within various regions of the brain including the hippocampus and cerebellum.[93] Both IDO and TDO have however been shown to be expressed at substantially lower levels

within the brain than in the periphery making the concentration of kynurenines within the brain largely influenced by those transported across the BBB from the periphery.[94] Tryptophan, as well as the peripheral kynurenines, KYN and 3-HK are readily transported across the BBB via specialized transporters. Once in the brain, these TRP metabolites may become further degraded to produce the kynurenines KYNA and QUIN, which do not easily cross the BBB from the periphery (making within brain levels largely dependent on the synthesis from these kynurenine precursors).[95] KYNA and QUIN are synthesized along distinct pathways within the brain due to their reliance on their respective KAT and KMO enzymes. Astrocytes possess KAT while lacking KMO thereby allowing them to participate only in the conversion of KYN to KYNA. Alternatively, microglia possess the enzyme KMO, allowing them to convert KYN to 3-HK which is later converted to QUIN via a spontaneous reaction further downstream.[96] An appropriate balance between these 2 branches of the kynurenine pathway is therefore critical in the maintenance of appropriate QUIN and KYNA levels within the brain.

Under healthy conditions the peripheral component of the kynurenine pathway is well regulated, resulting in a healthy balance between peripheral concentrations of TRP and its kynurenine metabolites. As such, appropriate levels of TRP and its BBB transportable metabolites, KYN, and 3-HK are available for transportation into the brain. This allows for the synthesis of adequate levels of the neurotransmitter 5-HT, and the neuroactive kynurenines KYNA, 3-HK, and QUIN, within the brain.

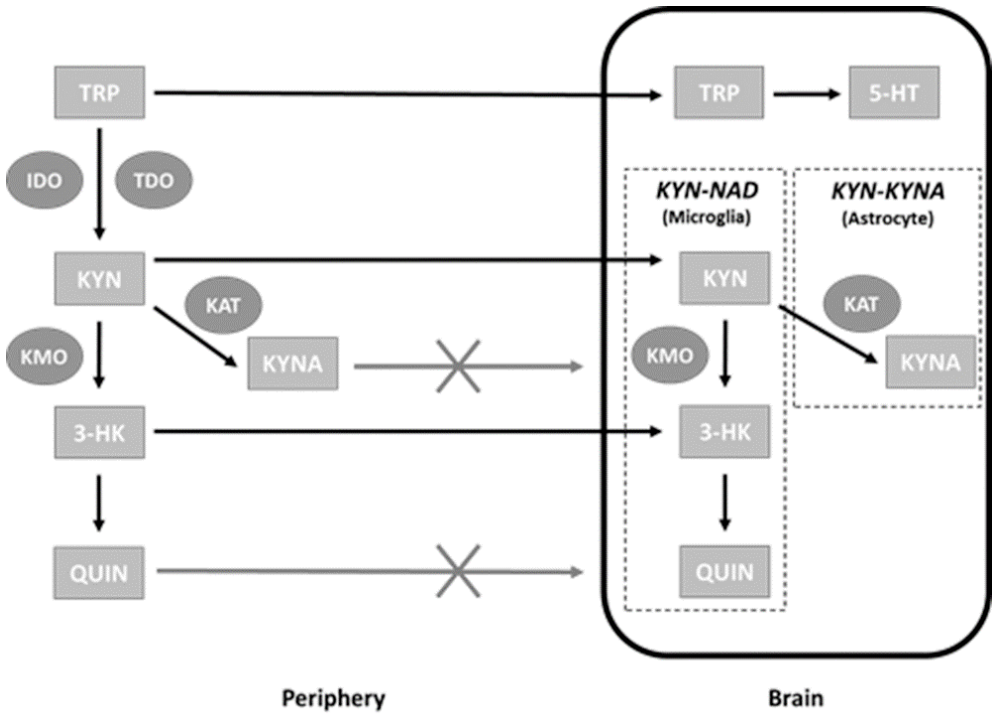

Figure 2: Peripheral and central kynurenine pathway interaction

As indoleamine 2,3-dioxygenase (IDO) and tryptophan 2,3-dioxygenase (TDO) are found at only very low concentrations within the brain, concentrations of within-brain tryptophan (TRP) and kynurenines are largely dependent on those from the periphery. TRP from the periphery competes with other large neutral amino acids (LNAA) for passage across the blood brain barrier (BBB) to be used in the synthesis of serotonin (5-HT). Kynurenine (KYN) and 3 hydroxykynurenine (3-HK) are also capable of crossing the BBB whereby they participate in the production of kynurenic acid (KYNA) and quinolinic acid (QUIN) which do not easily cross it.

The Kynurenine Pathway and Chronic Inflammation

During a typical immune challenge, the body responds with an acute elevation in peripheral proinflammatory cytokines. Certain cytokines, such as interferon gamma (IFN-y), up-regulate IDO activity, thereby causing an elevation in TRP degradation and an increased production of kynurenines.[97,98] As various parasites, viruses, and bacteria rely on TRP to grow and spread throughout the body, this acute response is a crucial adaptive mechanism meant to reduce TRP availability and thereby limit the spread of the pathogen.[97] During a healthy immune response, the elevation of proinflammatory

cytokines is followed by an elevation in anti-inflammatory cytokines, acting to restore a balance in inflammatory mediators.

However, under a state of chronic inflammation, proinflammatory cytokines are maintained at a perpetually elevated state. This results in the chronic up-regulation of IDO leading to a potential lasting shift in the kynurenine pathway. This shift may lead to dramatic reductions in peripheral TRP availability as well as a surplus of peripheral kynurenines.[2] Elevated concentrations of kynurenines such as KYN, 3-HK, and QUIN have been shown to suppress T-cell proliferation and inhibit the production of TH1 cytokines.[99,100] Such an effect may partly explain the impact that TRP metabolism has on immunity under pathological conditions. A reduction in peripheral TRP concentrations may also result in a TRP deficit within the brain, thereby leading to reduced levels of 5-HT synthesis. In addition, TRP metabolites within the brain may become elevated via several mechanisms. First, the surplus of peripheral kynurenines may result in elevated levels of KYN and 3-HK within the brain due to their ability to cross the BBB. Second, during an inflammatory response, macrophages, which house key enzymes, have the ability to infiltrate the brain and thereby participate in the production of kynurenines. Lastly, as cytokines are able to cross the BBB they are able to up-regulate corresponding enzymes within astrocytes, microglia and invading macrophages resulting in an even further up-regulated production of kynurenines.[101] Together, these inflammatory based mechanisms may result in a 5-HT deficit and surplus of kynurenines within brain which have important implications in symptoms of both depression and cognitive impairment.

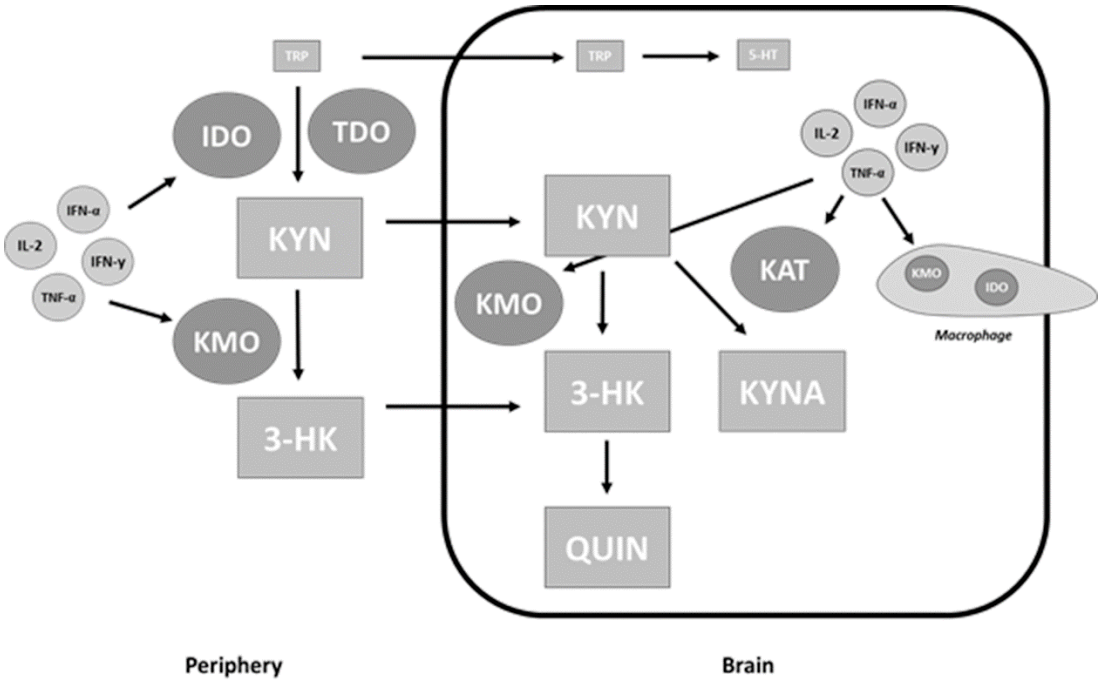

Figure 3: Influence of chronic inflammation on the kynurenine pathway

Certain proinflammatory cytokines possess the ability to up-regulate enzymes of the kynurenine pathway, thereby increasing the rate of tryptophan (TRP) degradation and production of kynurenines in the periphery. This may result in reduced concentrations of peripheral TRP and elevated peripheral levels of kynurenines. The reduced levels of peripheral TRP may then result in insufficient levels within the brain and a corresponding serotonin (5-HT) deficit. Proinflammatory cytokines also up-regulate enzymes in the brain, housed within astrocytes, microglia, and infiltrating macrophages, which utilize the elevated kynurenine concentrations for the production of further metabolites such as quinolinic acid (QUIN) and kynurenic acid (KYNA).

Depression

An elevated inflammatory status may influence depressive symptoms via several potential mechanisms related to neuroactive compounds of the kynurenine pathway. First, proinflammatory cytokines such as IFN-y, interferon alpha (IFN-α), interleukin-1 beta (IL-1β) and tumour necrosis factor alpha (TNF- α) have each been shown to directly up-regulate serotonin transporter (SERT) proteins within the brain leading an increased reuptake of 5-HT and a corresponding reduction in extracellular concentrations.[102,103] Second, proinflammatory mediators may influence depressive symptoms by means of

several indirect mechanisms associated with alterations in the activity of enzymes of the kynurenine pathway.

The up-regulation of IDO and TDO and the resulting increased rate of TRP degradation may result in a TRP deficit within the periphery. This may lead to an insufficient level of TRP transportation across the BBB and ultimately contribute to a 5-HT deficit within the brain. The importance of maintaining appropriate peripheral levels of TRP have been evidenced by a number of studies in which reductions of 5-HT synthesis and relapses in depressive symptoms have been demonstrated following the transient reduction of TRP levels by means of dietary restriction.[104–108] In order to be utilized in the synthesis of 5-HT, peripheral tryptophan must compete with other large neutral amino acids (LNAA) to cross the blood brain barrier via a common transport mechanism. As an over-activation of IDO and TDO induces a selective decline in peripheral tryptophan levels, a reduction in the peripheral TRP/LNAA ratio results, thereby reducing TRP availability for the synthesis of 5-HT.[87] Therefore under conditions of chronically elevated levels of proinflammatory cytokines the resulting alterations in enzyme activity and peripheral tryptophan levels may contribute to a 5-HT deficit within the brain.

The up-regulation of IDO and TDO also results in an elevated peripheral concentration of 3-HK and QUIN. Peripheral 3-HK is able to cross the BBB and induce oxidative damage via the production of reactive oxygen species (ROS) following an interaction with the enzyme xanthine oxidase.[109] Additionally, 3-HK may be further metabolized within microglia along the KYN-NAD branch of the kynurenine pathway to produce QUIN within the brain. QUIN is a potent agonist of a glutamatergic receptor

known as the N-methyl-D-aspartate (NMDA) receptor. These receptors are heavily concentrated on the hippocampus and play an important role in synaptic plasticity. When at elevated concentrations, QUIN is capable of inducing excitotoxicity by causing an over-activation of NMDA receptors leading to an increased influx of calcium (Ca^{+}) and corresponding neuronal damage.[110] This can also lead to the production of additional free radicals and further contribute to the oxidative stress brought on by 3-HK. In this way, both 3-HK and QUIN may contribute to neurodegeneration associated with depression.

The ability of elevated QUIN concentrations to cause an over-activation of NMDA receptors may also contribute to the hippocampal atrophy and hypothalamic-pituitary-adrenal (HPA) axis over-activity commonly reported in individuals with major depression.[111–115] The hippocampus plays an integral role in the attenuation of the HPA axis via a glucocorticoid induced negative feedback loop. When glucocorticoids, such as cortisol, are released by the adrenal glands they act on corresponding receptors of the hippocampus, resulting in an inhibitory cascade within the HPA axis. Specifically, the activation of glucocorticoid receptors on the hippocampus results in a blunted release of corticotropin releasing hormone (CRH) from the hypothalamus, which inhibits the release of adrenocorticotropic hormone (ACTH) from the pituitary, ultimately reducing glucocorticoid release from the adrenal gland. However, overstimulation of NMDA receptors may result in hippocampal atrophy and a corresponding loss of glucocorticoid receptors. This may result in the loss of negative feedback and HPA axis over-activity. An elevation in QUIN levels within the brain as well as elevations in systemic cortisol levels may therefore contribute to these aspects of depression. Elevated levels of both QUIN and cortisol have been demonstrated in individuals with depression.[116,117]

In addition to these proposed mechanisms, several pre-clinical and clinical lines of evidence support the relationship between proinflammatory cytokines and depression. Individuals diagnosed with major depression have been consistently reported to demonstrate elevated levels of proinflammatory cytokines.[27,28,89] Evidence that such elevations contribute to depression via an IDO related mechanism has also been demonstrated following cytokine therapy, utilized during human cancer trials. The acute administration of the pro-inflammatory cytokines, interleukin-2 (IL-2) and interferon- α (IFN- α), was shown to induce an increase in IDO activity and a corresponding reduction in both the peripheral TRP/LNAA ratio and absolute TRP concentrations.[118] Such changes in enzyme regulation and protein balances have been shown to result in the development of major depressive disorders in 15-40% of patients undergoing cytokine therapy with IFN-α.[119] As such, a state of chronic inflammation associated with an elevation in inflammatory mediators may contribute to symptoms of depression by means of various mechanisms associated with imbalances in the kynurenine pathway.

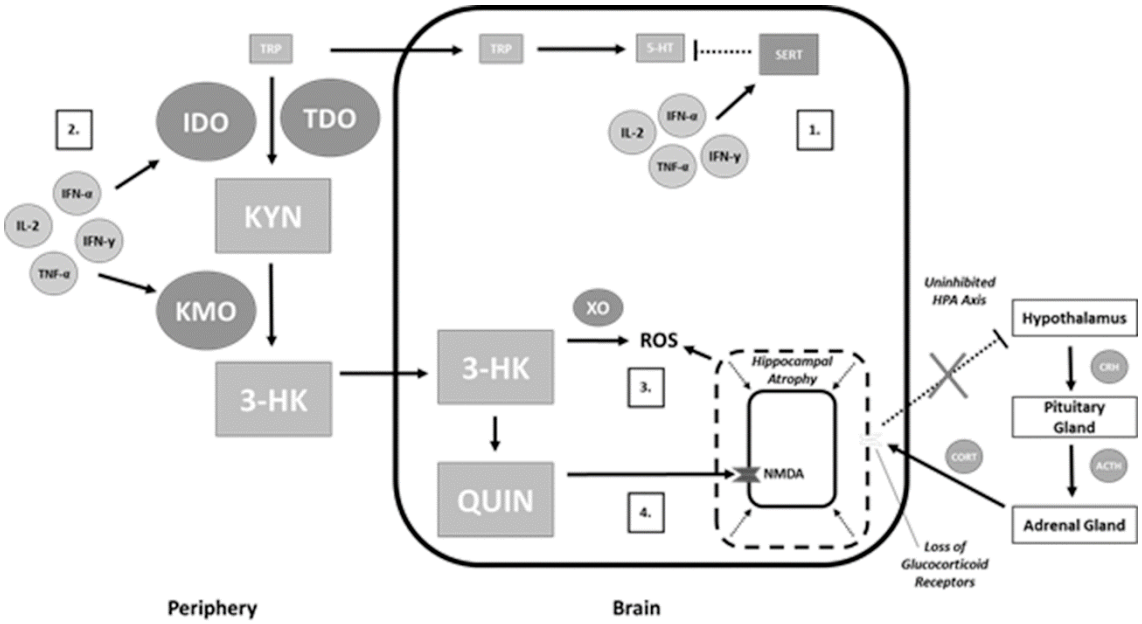

Figure 4: Inflammatory mechanisms of depression

Proinflammatory cytokines may contribute to depressive symptoms by means of various mechanisms. (1) Proinflammatory cytokines act on serotonin transporter (SERT) proteins within the brain causing a re-uptake of serotonin (5-HT) and corresponding reduced extracellular concentrations. (2) Proinflammatory cytokines up-regulate enzymes such as indoleamine 2,3-dioxygenase (IDO) and tryptophan 2,3-dioxygenase (TDO) resulting in reduced tryptophan (TRP) availability, ultimately contributing to reduced 5-HT synthesis. (3) Both 3-hydroxykynurenine (3-HK) and quinolinic acid (QUIN) may contribute to elevated levels of reactive oxygen species (ROS) and oxidative stress within the brain. (4) QUIN may induce N-methyl-D-aspartate (NMDA) over-activity thereby contributing to hippocampal atrophy and a loss of glucocorticoid receptors, ultimately leading to a loss of negative feedback and hypothalamic-pituitary-adrenal (HPA) axis over-activity.

Cognitive Impairment

Cognitive impairment has been commonly demonstrated in depressed individuals and the severity of deficits have been shown to correlate with the number of depressive episodes experienced.[120] The relationship between depression and cognitive impairment is not fully understood, however, they may share a closely linked inflammatory etiology. The influence of chronic inflammation on hippocampal volume and HPA axis dysregulation may play an important role in the severity of each of these disorders.

Hippocampal volume loss in the form of reduced grey matter density has been demonstrated in depressed individuals and has been shown to correlate with reduced scores in verbal recognition memory.[121] As previously discussed, there is evidence to support a role for chronic inflammation in the reduction in hippocampal volume involving heightened levels of metabolites and steroid hormones such QUIN and glucocorticoids. The potential apoptosis and/or inhibition of neurogenesis caused by over-activation of the hippocampus would be expected to result in cognitive deficits, due to the integral role the hippocampus plays in learning and memory. It may also lead to a vicious cycle whereby the loss of hippocampal-mediated inhibition of the HPA axis results in excess glucocorticoid production, ultimately further contributing to the extent of hippocampal atrophy.

In addition to the potential structural damage, the shift in the kynurenine pathway, brought on by chronic inflammation, may also contribute to cognitive deficits, via reductions in neurotransmitter release. Elevated concentrations of KYN within the brain result in its metabolism along one of the two distinct branches of the kynurenine pathway. The KYN-NAD branch results in the production of 3-HK and QUIN which have implications in the production ROS and excitotoxicity (as previously discussed). However, the primary enzyme of the KYN-NAD branch, KMO, has been shown to be far less active in the brain in comparison to the periphery, and as such, becomes rapidly saturated by elevated levels of KYN.[122] This may result in a shift in the kynurenine pathway towards the KYN-KYNA branch and an increased production of KYNA by the more active KAT enzyme.

KYNA acts as an antagonist of the alpha-7-nicotinic acetylcholine receptor (α7nAChR) and to a lesser extent, the glycine co-agonist site of the N-metheyl-D-asparate receptor (NMDAR).[123] Each of these receptors can be found on the hippocampus and are known to play important roles in synaptic plasticity, associate with learning and memory.[124] The inhibition of α7nACh receptors by KYNA has been shown to result in the reduced release of neurotransmitters such as glutamate, acetylcholine, and dopamine; each of which play critical roles in cognitive processes.[125–127] Additionally, the reduction of KYNA has been demonstrated to enhance extracellular glutamate and corresponding cognitive behavior[124]

This relationship has been demonstrated using animal models whereby elevations of KYNA within the brain have been shown to induce cognitive deficits in contextual learning and memory. Whether induced indirectly, via intraperitoneal administration of kynurenine, or via direct intracerebroventricular KYNA infusion, corresponding elevations in KYNA levels within the brain have been shown to induce spatial working memory deficits and reduced orienting behavior in mice.[128,129] Similar results have been demonstrated in healthy humans whereby administration of the non-competitive NMDA glutamate receptor antagonist, ketamine, has been shown to result in reductions in verbal declarative memory.[130] Alternatively, animal models displaying low levels of KYNA have demonstrated superior cognitive performance. This has been shown using kynurenine aminotransferase II (KAT II) knock-out mice, who lack the major enzyme for brain KYNA formation. These mice exhibited a 66% reduction in extracellular KYNA accompanied by a significantly increased performance in object exploration and recognition, passive avoidance, and spatial discrimination.[124]

A shift in the kynurenine pathway as well as corresponding elevated levels of inflammatory mediators have also been demonstrated in humans afflicted with conditions associated with severe cognitive deficits. Individuals with Alzheimer's disease have been shown to exhibit reduced peripheral tryptophan concentrations, along with heightened levels of the tryptophan metabolite, QUIN.[131] Additionally, severe elevations, of up to 25-fold, of the proinflammatory cytokine TNF-α have been demonstrated.[132] As TNF-α is a proinflammatory cytokine and potent IDO activator, it may suggest an inflammatory contribution to the molecular imbalances observed in this population. Further to these observations, the administration of the TNF-α antagonist, etanercept, has been shown to result in improved cognitive scores over a 6-month administration period as well as acutely following a single dose.[132,133] Such improvements provide further evidence of a role for inflammatory mediators in cognitive processes via alterations in enzyme regulation and corresponding imbalances of critical neuroactive compounds.

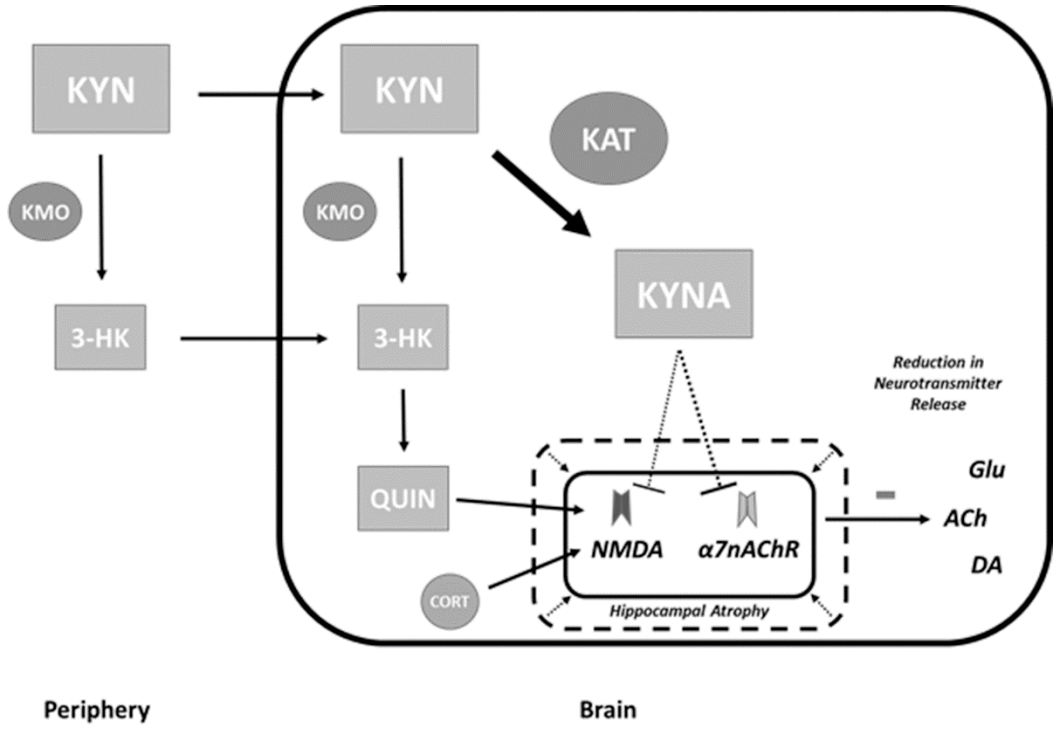

Figure 5: Inflammatory mechanisms of cognitive impairment

Hippocampal atrophy caused by the over-activation of respective receptors for glucocorticoids and quinolinic acid (QUIN) may contribute to cognitive impairment. Additionally, elevated concentrations of kynurenine (KYN) that is preferentially metabolized along the kynurenine-kynurenic acid (KYN-KYNA) branch of the kynurenine pathway results in elevations of kynurenic acid (KYNA). As KYNA acts as an antagonist for both the alpha-7-nicotinic acetylcholine (α7nACh) and N-methyl-D-aspartate (NMDA) receptors it may contribute to cognitive deficits by reducing neurotransmitters such as glutamate, acetylcholine, and dopamine.

The Inflammatory Etiology of Neuropathic Pain

Nociceptive pain reflects our capacity to detect potentially harmful stimuli. This sensation is mediated in the periphery by sensory neurons known as nociceptors which transmit information through nociceptive pathways in the spinal cord to the brain. Peripheral tissue injury/inflammation causes reversible changes to this sensory system causing pain hypersensitivity whereby reduced thresholds at the peripheral nerve terminals of nociceptors allow mechanical, thermal, and chemical stimuli to be converted

into voltage potentials. This process is beneficial as ensures any contact or overuse of the damaged area is avoided until healing has occurred. However, when the nervous system itself is injured, neuropathic pain may result whereby threshold changes are persistent or permanent and allodynia and/or hyperalgesia may result.[134] In this case, nerve lesions result in the heightened peripheral sensitization of nociceptors leading to ectopic firing of injured sensory axons. Traditionally, this phenomenon has been considered a purely neuronal response, however, it is now well established that supporting glial cells and the environment with which the nociceptor interacts are also important factors in neuronal function.[135] Evidence of this has been demonstrated as alterations in ion channel trafficking and expression at the nociceptor nerve terminal have been shown to occur in both injured and uninjured sensory fibers.[136]

Chemicals which surround peripheral nerve terminals of nociceptors determine baseline sensitivity and activation thresholds.[135] In this way, inflammatory mediators such as cytokines can sensitize nociceptors such that they respond to normally innocuous thermal and mechanical stimuli.[135] Proinflammatory cytokines such as IL-1B, IL-6, and TNF-α have been proposed to induce algesic effects by means of both indirect and direct influences on nociceptors.[137] Indirectly, cytokines can reduce nociceptive thresholds in a prostaglandin-dependent manner. Proinflammatory cytokines cause the up-regulation of the enzyme COX which leads to increased gene transcription of further mediators such as NO and PGE2. These mediators are believed to induce algesic effects through their ability to sensitize nociceptors by acting directly on receptors on the neuronal cell membrane.[88] A potential direct neural influence of cytokines has also been suggested due to the rapid onset of pain following their administration in animal models. For example,

injection of IL-1B excites nociceptive fibers within 1 minute of administration, indicating a more direct action then the aforementioned prostaglandin dependent mechanism.[137] IL-1B has also been implicated in neuropathic pain mechanisms based upon the significant reduction in mechanical hypersensitivity in mice with a genetic impairment of IL-1B.[138,139]

The overall outcome of human chronic pain states may correlate strongly with the balance between pro and anti-inflammatory cytokines. Studies have shown that individuals with complex regional pain syndrome, painful neuropathy, and SCI have systemic increases in pro-inflammatory cytokines TNF, IL-6, and IL-2, while anti-inflammatory cytokines IL-10 and IL-4 are reduced.[13,29] Alternatively, studies examining subjects with painless neuropathies have shown elevated levels of anti-inflammatory cytokines.[140] There is also evidence in animal models that blockade of proinflammatory cytokines or administration of anti-inflammatory cytokines reduces neuropathic hyperalgesia,[141–143] whereas administration of the proinflammatory cytokines IL-1B and TNF reduce nociceptive thresholds and induce further pain.[144] Furthermore, proinflammatory cytokines induce the production of one another which may lead to a positive feedback loop of increasing chronic inflammation and pain if not adequately suppressed.

Therefore, anti-inflammatory treatments to help shift the TH1:TH2 balance whereby proinflammatory cytokines are reduced and anti-inflammatory cytokines are increased may be an effective strategy for treatment chronic neuropathic pain.

The Inflammatory Etiology of Neural Deficits

At highly elevated concentrations certain cytokines (eg. TNF-α) have been shown to directly influence nerve conduction in a reversible dose dependent manner.[34] This has been demonstrated using spinal cord tissue in ex-vivo animal models. Although no direct evidence of such an effect occurring in humans has been sufficiently demonstrated, indirect evidence exists to suggest this potential role. In a case study performed by Mcdonald et al. (2002), a complete tetraplegic was examined 5 years post injury upon beginning an intensive diet and exercise program.[145] After undergoing no observable changes for the initial 5 years post injury, the initiation of the diet and exercise program resulted in a dramatic reduction in secondary health complications over the following several years. Interestingly, the reduction in infections was matched by significant improvement in both motor and sensory scores. The improvement in sensation is of particular interest as this cannot be considered a direct result of the training itself. It is important to note that that this study did not assess inflammatory mediators. However, it is possible to speculate that the reduction in secondary health complications would have in all likelihood led to a reduction in inflammatory mediators. Such reductions may be partly responsible for the improvements in somatic nerve conduction via mitigation of similar channelopathic effects as suggested in the study by Davies et al. (2006).

Therefore, depending on the severity of the effect cytokines and other inflammatory mediators may have on somatic nerve function, anti-inflammatory interventions may have the potential to improve somatic nerve function which could even translate into improved motor and sensory function.

Treatment

Current Treatment Strategies

The majority of current treatment strategies aimed at improving symptoms of depression utilize drug treatments which target downstream enzymes, transporters, or receptors. Of these, selective serotonin reuptake inhibitors (SSRI) have become the most commonly used; due to their ability to induce a relatively strong and immediate alleviation of symptoms. Drug treatments of the SSRI class target serotonin transporters (SERT), and inhibit them from carrying out their role concerning 5-HT reuptake, thereby increasing extracellular levels. Use of SSRIs is however, associated with a number of side-effects and only provides transient relief of symptoms. They have also been shown to be ineffective in approximately 30% of patients [146], in whom a particularly elevated inflammatory state is typically reported [147,148]. Additionally, of individuals who do respond to treatment, an estimated 20-80% will relapse and experience a depressive episode within 1-5 years following initial treatment [149]. It may be possible that under extreme or reoccurring inflammatory episodes, the shift in the kynurenine pathway and resulting imbalance in neuroactive kynurenines, hippocampal damage and HPA axis dysfunction, may cause alterations too severe for SSRI treatment to remedy. This could partially explain the ineffectiveness of drug treatments such as SSRIs in individuals with a highly elevated inflammatory state and may suggest a need for the addition of anti-inflammatory interventions in the treatment of depression.

More recent attempts to treat cognitive impairment have focused on counteracting and/or limiting the antagonizing effects of KYNA. Agonists of the α7nACh and NMDA receptors such as galantamine have been utilized in an attempt to counteract the antagonizing role of KYNA. Such attempts have however, produced inconclusive and

only partially positive results [150–154]. It may be possible that elevated concentrations of KYNA lead to receptor saturation thereby making it difficult to achieve any benefit from such receptor agonists. In an alternative approach, early studies examining the use of selective inhibitors for the primary enzyme involved in KYNA production, KAT II, have shown positive results in animal models. The administration of KAT II inhibitors resulted in reductions in extracellular KYNA along with elevations in glutamate, dopamine, and acetylcholine [127]. Further studies are required to examine the effectiveness of such treatments in humans as well as an appropriate dosage. As α7nACh and NMDA receptor activity has important implications in both depression and cognitive impairment, maintaining the delicate balance between receptor agonists and antagonists is critically important. As such, if either receptor agonists or KAT II inhibitors were to be used in the long-term treatment of cognitive impairment, the dose would need to be strictly controlled in order to avoid NMDA over-activity and the possibility of hippocampal atrophy and HPA axis dysregulation over time. A safer alternative may be to naturally induce molecular alterations over time via anti-inflammatory intervention strategies.

There is currently no effective treatment to alleviate neuropathic pain. Patients with neuropathic pain do not respond to non-steroidal anti-inflammatory drugs (NSAIDs), commonly become resistant to opiates, and show little improvement paired with undesirable side-effects following treatment with anti-depressants.[155] The majority of current treatments therefore focus on psychological coping strategies rather than the elimination of pain.

Although the value of current drug treatments should not be discounted, alternative therapies which target the inflammatory basis of such disorders should also be

considered. Utilizing intervention strategies which target upstream proinflammatory mediators may help to restore proper enzyme regulation and induce corresponding beneficial alterations in neuroactive compounds, thereby positively influencing a variety of disorders. Simple lifestyle alterations including the adoption of diet consisting of foods and supplements with proven anti-inflammatory properties and participation in regular exercise may provide a safer, sustainable, and more universally applicable treatment option to that of traditional drug treatments. Although such intervention strategies do not provide the immediate effects of drug therapies, they may contribute to a more permanent solution while helping to reduce the many side-effects associated with a heavy reliance on drug interventions.

Exercise as an Anti-inflammatory Intervention

Regular moderate exercise has been consistently shown to reduce chronic low-grade inflammation and protect against a number of associated diseases [156,157]. Numerous cross-sectional studies have demonstrated reductions in inflammatory mediators within trained vs. untrained individuals[158–161]. Although the anti-inflammatory nature of regular exercise has been well established, the mechanisms by which this is accomplished are not fully understood and are likely the result of numerous exercise-related factors.

One such theory involves a unique exercise-induced inflammatory response which differs from that of the typical response evoked by infection. Whereas infection results in the initial elevation in proinflammatory cytokines, such as TNF-α and IL-1β, it has been suggested that during an exercise-induced inflammatory response, the elevation in proinflammatory cytokines is bypassed. Instead the initial response is a large elevation in interleukin-6 (IL-6); a cytokine with both pro and anti-inflammatory properties. This

spike in IL-6 is followed by an elevation in anti-inflammatory cytokines, such as interleukin-10 (IL-10) and interleukin-1 receptor antagonist (IL-1RA) [156,157]. It has been suggested that IL-6 promotes an anti-inflammatory environment during exercise by promoting IL-10 and IL-1RA while inhibiting TNF-α production. This IL-6-induced inhibition of TNF-α has been demonstrated *in-vitro,*[162] as well as *in-vivo* in animal models [163], and in humans [164]. Exercise may also inhibit proinflammatory mediators through IL-6-independent pathways via exercise-induced elevations in hormones such as epinephrine. This has been suggested due to the demonstration of TNF-alpha inhibition during exercise in IL-6 knockout mice [165], as well as the suppression of TNF-alpha following epinephrine infusion in-vivo [166]. Whether hormonally driven, or due to a unique influence from IL-6, if acute bouts of exercise promote an anti-inflammatory environment, it may explain how exercise, when performed on a regular basis, may act to protect against a chronic low-grade inflammatory state.

Of note, many studies have demonstrated that acute bouts of exercise do in fact result in a proinflammatory response characterized by leukocytosis and elevations in proinflammatory mediators [167–169]. An acutely elevated rate of TRP metabolism and resulting increase in serum kynurenine concentrations have also been demonstrated and have been suggested to relate to a cytokine-induced up-regulation of IDO [170,171]. Further, exercise is known to result in an acute elevation in glucocorticoids [172] which have been suggested to induce the up-regulation of TDO2, further contributing to the elevated rate of TRP metabolism during exercise [173]. Despite the proposed short-term proinflammatory response, long-term metabolic and cardiovascular training adaptations are associated with a chronic reduction in inflammatory mediators which likely override the acute

proinflammatory effects of exercise, resulting in a chronic shift towards an anti-inflammatory state. For example, adipose tissue is known to act as an endocrine organ, responsible for the release of a variety of proinflammatory mediators termed adipokines. As such, a reduction in adipose tissue, as would be expected over the span of a chronic training program, may result in a reduction in such proinflammatory mediators [174]. Corresponding improvements in vascular health may also protect against plaque formation seen in atherosclerosis leading to reductions in vascular inflammation [157]. In addition to these factors, individuals who participate in regular exercise are also likely to be non-smokers, less prone to hypertension, and have lower cholesterol levels, which have each been shown to be inversely correlated with C-reactive protein (CRP) concentrations[158].

Inconsistencies in the literature regarding the inflammatory response to an acute bout of exercise may be due to a lack of consistent exercise protocols. Differences in the mode, duration, and intensity of exercise bouts may lead to inconsistent levels of exercise-induced muscle damage, and metabolic demands resulting in variable levels of inflammatory mediators. It will be important to determine the most ideal exercise parameters to achieve the greatest inflammatory benefit if there are hopes of utilizing exercise as an effective treatment for inflammatory based disorders. This gap in the literature may also help explain inconsistencies regarding the degree to which exercise reduces symptoms of depression and cognitive impairment. Although there is a general consensus that exercise positively impacts mood and cognition, some studies show improvements equivalent to that of traditional medications [175,176], while others show that exercise is only mildly more effective than control conditions [177]. Additionally, the

mechanisms by which such benefits may be accomplished are widely debated, ranging from increased cerebral blood flow, to changes in neurotransmitter release, to actual structural changes in the central nervous system (CNS), such as the hippocampus [178]. Interestingly, each of these proposed adaptations can be influenced by the inflammatory response through mechanisms previously discussed. There is a need for more methodologically robust, prospective trials with consistent exercise protocols in order to examine the true potential of exercise as a treatment strategy following SCI. Nevertheless, the anti-inflammatory properties of regular exercise have been well-established, and in theory, it should help to improve or prevent symptoms related to depression and cognitive impairment by targeting upstream inflammatory processes. As such, despite the insufficient evidence to definitively state the degree to which exercise interventions may impact these disorders, it would seem there is certainly merit to implement them along with traditional drug treatments.

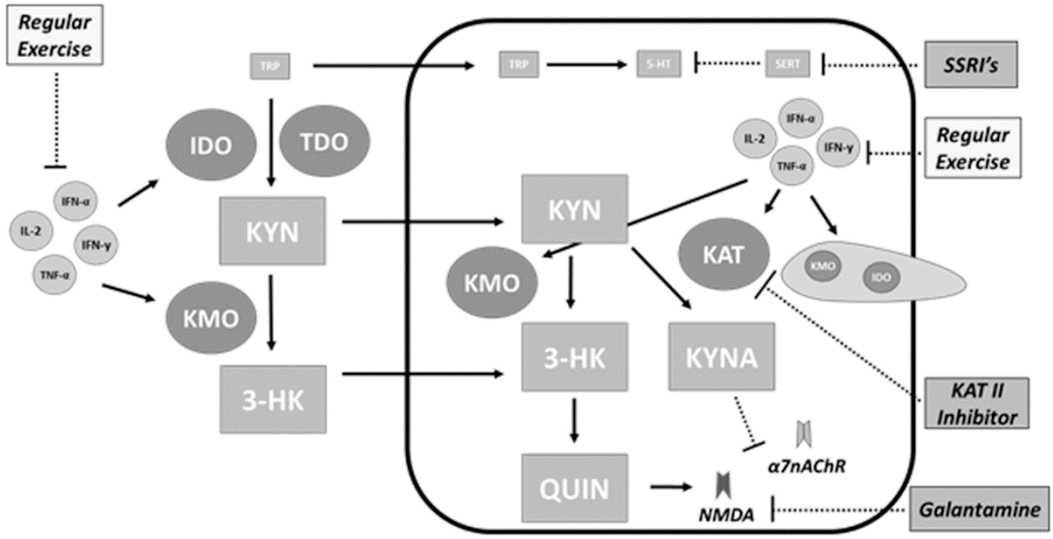

Figure 6: Targets of treatment interventions

Common treatment strategies for disorders associated with depression and cognitive impairment typically target downstream enzymes or receptors. Alternatively, intervention strategies such as regular exercise possess anti-inflammatory properties which helps restore a balance in inflammatory mediators, thereby restoring proper enzyme regulation and protein balances upstream.

Diet as an Anti-inflammatory Intervention

Diet may be used as a powerful tool for treating immune dysfunction and chronic inflammation. The consumption of foods and supplements with anti-inflammatory properties can induce beneficial effects by means of a variety of mechanisms including alterations in cell membrane composition, enzyme activity, and gene transcription. The intake of antioxidants has also been demonstrated to aid in the treatment of chronic inflammation via the reduction of free radicals necessary in oxidative burst and anti-microbial activities of phagocytes. The corresponding reduction in the redox ratio has also been shown to induce a reduced activation in NFkB thereby reducing gene transcription of inflammatory enzymes. Lastly, proper diet may aid in the reduction of inflammation via loss of adipose tissue resulting in a reduced production of

proinflammatory adipokines. Altering one's diet to include sufficient quantities of such nutrients and appropriate caloric intakes may aid in immune health by modifying the production of proinflammatory mediators and thereby helping to reestablish a more balanced immune state. Unfortunately, a lack of awareness, as well as the perceived cost and effort involved in maintaining a healthy diet commonly leads to nutrient insufficiencies in the general population as well as in those with SCI.

One such example of this pertains to the intake of polyunsaturated fatty acids (PUFAs). In a typical Western diet, omega-6 (n6) intake is on average much higher than that of n3. As such, these fatty acids are preferentially utilized in the composition of cell membranes and typically greatly predominate n3 concentrations. Specialized enzymes known as cyclooxygenase (COX) and lipooxygenase (LOX) act on these membranes and utilize their fatty acids to produce inflammatory mediators. When n6 is utilized as a substrate, potent proinflammatory molecules such as prostaglandin-2 (PGE2) and leukotriene4 (LTB4) are produced. These molecules (known as eicosanoids) produce proinflammatory effects such as increased chemotaxis, increased production of adhesion molecules, vasoconstriction, and pain.[179] However, when the COX and LOX enzymes utilize n3 as a substrate much less potent proinflammatory eicosanoids are produced such as prostaglandin-3 (PGE3) and leukotriene-5 (LTB5). These eicosanoids produce a far weaker proinflammatory response. The utilization of n3 may also result in the production of anti-inflammatory and inflammation resolving proteins known as resolvins.[179] These resolvins act to inhibit chemotaxis, suppress proinflammatory cytokines, and aid in the clearance of cellular debris. Therefore, increased n3 intake in the diet may help to reduce

inflammation by means of alterations in cell membrane composition resulting in the production of less potent proinflammatory eicosanoids and anti-inflammatory resolvins.

Omega-3 fatty acids are also capable of reducing inflammation by limiting gene transcription of inflammatory molecules and enzymes. By inhibiting the translocation of the transcription factor NFkB to the nucleus of cells, n3 can prevent the transcription of genes for proinflammatory cytokines as well as the COX and LOX enzymes. Through these effects n3 may help to aid in the reduction of the chronic low grade inflammatory response associated with many pathologies.[179]

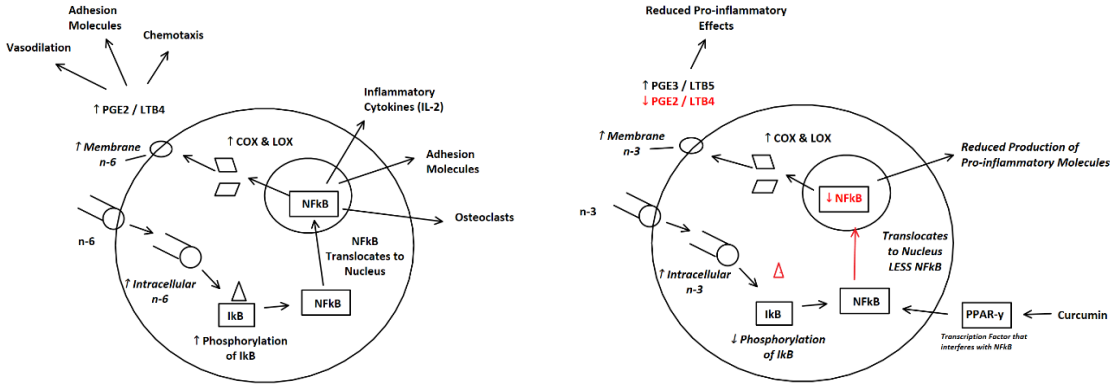

Figure 7: Intracellular inflammatory mechanisms of polyunsaturated fatty acids

Omega-6 acts intracellularly to increase phosphorylation of IkappaB proteins thereby enhancing translocation of NFkB to the nucleus of the cell where it can participate in the transcription of inflammatory mediators and enzymes. Omega-3 acts intracellularly to reduce phosphorylation of IkappaB proteins thereby limiting NFkB translocation to the nucleus of the cell and therefore reducing the production of inflammatory mediators and enzymes.

Various types of antioxidants induce anti-inflammatory effects via a number of mechanisms. Vitamin E consists of 4 different tocopherol homologues including α-tocopherol, β-tocopherol, У-tocopherol, and δ-tocopherol. Each homologue acts as an antioxidant whereby it may provide an electron to free radicals thereby converting them from their reduced state to their oxidized state. This helps to protect host tissue from

damage induced by free radicals but has also been suggested to play an inflammatory role via alterations in the redox ratio. When free radicals are converted from their reduced to oxidized state it causes a reduction in the redox ratio. This reduction has been shown to be associated with reduced activation of the transcription factor NFkB resulting in the reduced gene transcription for proinflammatory mediators and enzymes.[180] Therefore, tocopherols may help limit the inflammatory response not only by reducing levels of reactive oxygen species, but also by limiting NFkB activation.

Much like tocopherols, flavonoids also possess antioxidant and radical scavenging capabilities. In addition, flavonoids have been shown to possess their own mechanisms of anti-inflammatory activity in various animal models of inflammation.[181] Certain flavonoids have been shown to modulate enzyme activities such as the arachidonic acid (AA) metabolizing enzyme phospholipase A_2 (PLA_2) resulting in lower production of this omega-6 fatty acid. Flavonoids have also been shown to inhibit the enzymes COX and LOX resulting in reduced production of the potent proinflammatory eicosanoids PGE2 and LTB4.[182] Arachidonic acid, PGE2 and LTB4 are each crucial mediators of inflammation and the inhibition of their production would provide important anti-inflammatory effects.

Despite the known anti-inflammatory benefits of many naturally occurring nutrients and supplements, little attention has been paid to the utilization of an anti-inflammatory diet in the treatment of inflammation following SCI. The vast majority of studies which have examined the anti-inflammatory effect of supplements such as n3 have been performed on animal models or healthy human subjects. Of the few studies that have been performed on humans with chronic inflammatory conditions the focus has

typically been placed on subjects with rheumatoid arthritis. These studies have successfully demonstrated reduced pain and swelling, as well a reduction in proinflammatory mediators following n3 supplementation.[183,184] In spite of these positive findings, the use of anti-inflammatory diets as a potential treatment in the SCI population have been largely neglected. As an inflammatory basis is being established for an increasing number of disorders associated with SCI, such an intervention is worthy of examination.

Conclusion

As an inflammatory basis is becoming evident for a growing number of disorders, therapies which target the immune system and act to restore a balance in inflammatory mediators should be considered as potential methods of treatment and prevention. Although the effectiveness of current drug treatments should not be undervalued, pairing them with intervention strategies which target upstream inflammatory influence, may enhance the outcome of drug treatments for disorders impacted by chronic inflammation. Being that SCI is typically characterized by chronic inflammation as well as a higher prevalence of secondary neural and behavioural disorders, SCI is one such population which may particularly benefit from such strategies. It is difficult to make definitive conclusions regarding the degree to which diet and exercise, as an anti-inflammatory strategy, could influence neural and behavioural disorders as previous inferences are largely based on observational studies with highly variable protocols. However, the inflammatory etiology behind such disorders, and anti-inflammatory mechanisms of diet and exercise have been well established and therefore, should in theory, provide some benefit. Additionally, given the minimal side effects associated with such lifestyle

alterations, as well as the potential for a variety of other health benefits, there is seemingly little reason not to promote diet and exercise as treatment options. Whether these options have the potential to be effective as a stand-alone treatment, or would need to be utilized in conjunction with common drug treatments, is currently unclear and will likely vary between patients depending upon a number of factors. Further research will also be needed to determine the most appropriate exercise parameters in order to achieve the greatest anti-inflammatory benefit. A greater emphasis on lifestyle intervention strategies which target inflammation may provide stronger and more sustainable improvements by targeting the inflammatory mediators initially responsible for the alterations in enzyme regulation which ultimately contribute to a number of disorders. Such strategies may help to reduce disorders associated with SCI such as depression, cognitive impairment and neuropathic pain while avoiding the many side-effects associated with a heavy reliance on current drug treatments. If inflammatory mediators are proven to negatively influence somatic and/or autonomic nerve function, such anti-inflammatory strategies may even prove to benefit motor, sensory, and autonomic function in this population.

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Chapter 3

Purpose & Hypotheses

Overall Purpose & Hypotheses

The purpose of this study was to evaluate how reductions in chronic inflammation by means of an anti-inflammatory diet, in those with a SCI, may influence disorders with an established inflammatory basis including: depression, cognitive dysfunction, neuropathic pain, and somatic nerve deficits.

It was hypothesized that a diet focusing on foods and supplements with anti-inflammatory properties would be sufficient to significantly reduce levels of proinflammatory mediators such as cytokines and eicosanoids. Certain proinflammatory cytokines are capable of up-regulating key enzymes known to play a role in neuroimmune communication by means of altering pathways of protein catabolism. Therefore, the reduction of chronically elevated levels of proinflammatory cytokines was expected to result in beneficial alterations in both enzyme regulation, and levels of neuroactive compounds. If sufficient alterations were achieved, it was expected to translate into improvements in each of the disorders of interest.

Manuscript 1: Targeting inflammation to influence mood following spinal cord injury: A randomized clinical trial

The purpose of this study was to assess the relationship between mood and inflammation in individuals with SCI. It was hypothesized that by targeting the immune system and reducing levels of chronic inflammation it would be possible to induce corresponding changes in neuroactive compounds and improvements in mood and symptoms of depression.

Manuscript 2: Targeting inflammation to influence cognitive function following spinal cord injury: A Randomized Clinical Trial

The purpose of this study was to assess the relationship between cognitive function and inflammation in individuals with SCI. It was hypothesized that by targeting the immune system and reducing levels of chronic inflammation it would be possible to induce corresponding changes in neuroactive compounds and improvments in verbal learning and memory.

***Manuscript 3*: Targeting Inflammation as a Treatment Modality for Neuropathic Pain in Spinal Cord Injury: A Randomized Clinical Trial**

The purpose of this study was to assess the relationship between neuropathic pain and inflammation in individuals with SCI. It was hypothesized that by targeting the immune system and reducing levels of chronic inflammation and eicosanoids, it would be possible to induce corresponding reductions nociceptor sensitization and scores of neuropathic pain.

***Manuscript 4*: Targeting inflammation to influence peripheral somatic nerve function following spinal cord injury: A Randomized Clinical Trial**

The purpose of this study was to assess the relationship between somatic nerve function and inflammation in individuals with SCI. It was hypothesized that by targeting the immune system and reducing levels of chronic inflammation, it would be possible to improve nerve conduction velocity and signal amplitude in both motor and sensory nerves.

Chapter 4

Overview of Study Design

Study Design and Participants

The study was a randomized, parallel-group clinical trial. Participant recruitment occurred between September and November 2014. The study intervention was 12 weeks and included testing at baseline, 1 month, and 3 months. Participants with various levels and severities of SCI were recruited for participation in the study. Inclusion criteria included (1) over the age of 18, (2) SCI of any level or severity (American Spinal Injury Association A-D), (3) at least 2 years post injury. Exclusion criteria included (1) any contraindications to supplements provided in the study, (2) unstable dosage of any anti-depressive medications, (3) unstable medical condition within 2 weeks prior to intervention (4) pregnancy, (5) breastfeeding. Participant characteristics are shown in Table 1. Twenty individuals [10 male, 10 female; age 48.7±13.9yrs] with chronic [4-37yrs post-injury] SCI [C2-L4; ASIA Impairment Scale (AIS) A-D] were recruited for participation in the study. Twelve participants were randomly allocated to the treatment group, and were placed on the 12-week anti-inflammatory diet intervention, while 8 were allocated to the control group and received no intervention. Informed consent was obtained from all participants. The study was registered as a clinical trial (clinicaltrials.gov identifier: NCT02099890) and received ethical approval from the Brock University Research Ethics Board as well as the Natural Health Products Directorate of Canada. All data was collected on-site at Brock University and the Brock-Niagara Center for Health and Well-being.

Randomization

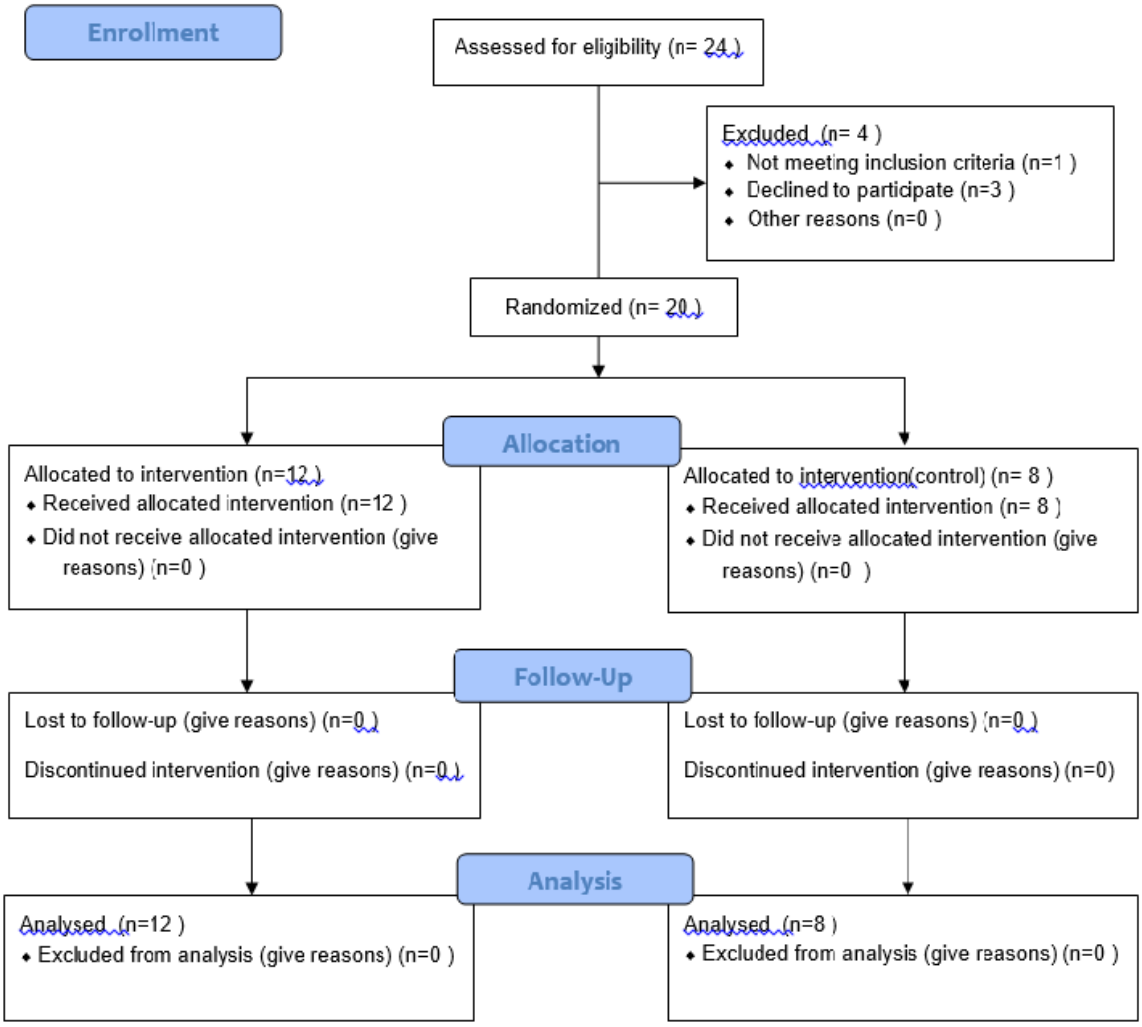
Randomization was computer generated by the primary investigator and stratified by participant sex and age using permuted blocks of 2 (male/female) and block of 3 (<40, 40-60, >60yrs). Randomization was 3:2 to either the anti-inflammatory diet vs control.

Table #1: Participant Characteristics

Participant	Sex	Age	AIS Score	Level of Injury	Time Since Injury (yrs)
Treatment					
1	F	44	D	C5	10
2	M	58	B	T10	4
3	F	62	D	L3	4
4	F	37	A	T3	19
5	M	22	A	C7	5
6	M	67	C	C2	4
7	M	66	D	C5	6
8	F	44	A	C7	9
9	F	65	D	T6	4
10	F	64	D	C3	37
11	M	45	A	T6	28
12	M	37	C	C4	23
AVE	--	51.5	--	--	12.8
SD	--	15.3	--	--	11.3
Control					
13	F	30	B	C5	6
14	F	63	D	L4	2
15	M	42	A	C5	6
16	F	58	D	C5	33
17	M	59	D	T4	4
18	F	33	A	T1	17
19	M	41	C	C4	22
20	M	36	A	C5	19
AVE	--	45.3	--	--	13.6
SD	--	12.9	--	--	10.9
p-value		0.38			0.87

**AIS: ASIA (American Spinal Injury Association) Impairment Scale*

Figure 8: CONSORT 2010 Flow Diagram



Flow diagram of participant recruitment and allocation

Dietary Intervention Protocol

The anti-inflammatory diet intervention focused on the elimination of common food intolerances and inflammation-inducing foods, as well as the introduction of foods and supplements with established ant-inflammatory properties. Examples of foods removed from the diet included those with high glycemic indices (such as refined wheat products and refined sugars), common intolerances such as cow's milk, and foods which negatively influence cardiovascular health such as hydrogenated fats. Participants also consumed daily supplements with established anti-inflammatory benefits. Omega-3 fatty acids (Now Ultra omega-3) were taken in softgel form, containing 500mg EPA and 250mg DHA, at a dosage of 3 pills per day. Chlorella (Now chlorella) was taken in pill form, containing 1000mg, at a dosage of 6 pills per day. Antioxidants (CanPrev antioxidant network) were taken in pill form, containing 100mg coenzyme Q10, 200mg n-acetyl-cysteine, 150mg mixed tocopherols, 100mg DL alpha lipoic acid, 60mg green tea extract, 5.5mg zinc, and 100ug selenium, at dosage of 2 pills per day. Curcumin (AOR Inflanox) was taken in pill form, containing 400mg, at a dosage of 3 pills per day. A vegetable based (yellow pea, brown rice, hemp seed) protein powder (Progressive Vegessential) containing 27g of protein was taken at a dosage of one scoop each morning.

Both the treatment group and control group were asked to complete a detailed diet record for 7 days at baseline, as well as 3 days at 3-months. The treatment group also recorded a 3-day diet record at 1-month, 2-months. This allowed for the examination of baseline eating habits as well as the assessment of compliance throughout the intervention. Food intake was assessed using The Food Processor (ESHA Inc. 2014, version 10.14.2, Salem, OR). Compliance to the specific anti-inflammatory diet was also

assessed by a detailed analysis of all diet records. Each food item was categorized as either a ‘food to consume’ a ‘food to avoid’ or a ‘neutral food’ based on the parameters of the diet participants were instructed to follow. Food was also categorized into servings in accordance with Canada’s Food Guide. Therefore, compliance scores were based on standard servings of foods subjects were instructed to eat vs. foods they were instructed to avoid. To account for differences in total energy intake, compliance scores were expressed as a ratio of the servings of foods to consume over the total servings of food (avoid + consume) multiplied by 100. This yielded a measure of percent compliance.

At the start of the study, the treatment group underwent an information seminar which explained the diet program followed by a one-on-one consultation with nutritionists during which their diet records were reviewed in detail and necessary changes were discussed. Participants received information regarding foods to eat and avoid, a supplement intake schedule and all necessary supplements, and list of approved recipes. Participants in the treatment group received support via weekly phone calls from members of our research team as well as a monitored online support group whereby participants could share recipes and experiences with one another. Participants in the control group were asked to maintain their current diets throughout the duration of the study.

Outcome Measures

All outcome measures were assessed at baseline, 1 month and 3 months. This allowed for the assessment of each of the outcome measures, under chronically elevated levels of proinflammatory mediators at baseline, as well as reduced levels of proinflammatory mediators at 1 and 3 months. Each testing session required a duration of

2 hours and included the assessment of: depression, cognitive function, neuropathic pain, and somatic nerve function. Blood draws were also performed for the quantification of associated inflammatory mediators and neuroactive compounds.

Quantification of serum inflammatory markers and amino acids

Blood draws (20ml) were taken from the antecubital vein of each participant at 1pm at each of the 3 testing sessions (baseline, 1 month, and 3 months). Following extraction, the whole blood was allowed to clot for 30 minutes followed by centrifugation at 1000xg for 15 minutes. Serum was extracted and immediately stored at -80°C until later analysis. Inflammatory mediators of interest included: pro-inflammatory cytokines IL-2, IL-1β, IL-6, TNF-α, IFN-y, the acute phase protein CRP; anti-inflammatory cytokines IL-4, IL-10, and IL-1RA; eicosanoids PGE2, LTB4; and amino acids (AA) and AA metabolites tryptophan (TRP), tyrosine (TYR), phenylalanine (Phe), BCAA (leucine, isoleucine, valine) and kynurenine (KYN). Analysis of pro and anti-inflammatory cytokines was performed in triplicate via the Magpix Multiplex system (EMD Millipore, MA, U.S.A) and analyzed using Luminex software. CRP, PGE2, and LTB4 were analyzed in triplicate and quantified via enzyme-linked immunosorbent assay (R&D systems, Minneapolis, U.S.A.). TRP, Phe, TYR, BCAA, and KYN were analyzed in triplicate and quanitified via enzyme-linked immunosorbent assay (Immunodiagnostik, Bensheim, Germany; Labor Diagnostika, Nordhorn, Germany).

Assessment of Depression

Participants completed the Center for Epidemiological Studies Depression Scale (CES-D) at each of the 3 testing sessions, as means of assessing symptoms of depression. The questionnaire consisted of 20 items related to depression and participants

were asked to rate how often they experienced each item over the previous 7-day period. Ratings were based on a 4-point scale including, 'rarely or none of the time' (less than 1 day), 'some or a little of the time' (1-2 days), 'occasionally or a moderate amount of the time' (3-4 days), or 'most or all of the time' (5-7 days). Points for each item ranged from 0-3 depending on frequency and each item was summed for a total score ranging from 0-60 with higher scores indicating the presence of more symptomatology. A score of 0-15 indicates no depressive symptoms, a score of 15-21 indicates mild to moderate depressive symptoms, and a score of >21 indicates the potential for major depression. The CES-D has been shown to be a valid and reliable measure of depression in SCI[1].

Inflammatory mediators of interest specific to depression included the proinflammatory cytokines: IL-1, IL-1β, IL-2, IL-6, and IFN-y. These proinflammatory cytokines were chosen (particularly IFN-y) due to their known ability to up-regulate the enzyme IDO. The quantity and activity of IDO was also examined due to its role in TRP degradation to a variety of metabolites including kynurenine and quinolinic acid[2]. Lastly, the large neutral amino acids, TRP, leucine, isoleucine, valine, and TYR were examined. TRP plays a critical role as a precursor in 5-HT synthesis and the other large neutral amino acids act as competitive antagonists for transportation across the blood brain barrier. Therefore, the TRP/LNAA ratio may be utilized as an indication of TRP availability for 5-HT synthesis.

Assessment of Cognitive Function

Participants were asked to perform the California verbal learning test (CVLT) as an indication of episodic learning and memory. This test consisted of 2 word lists each consisting of 4 categories of 4 words, for a total of 16 words per list. The test began with

the learning trial whereby the examiner read aloud the first list (List A) of words. These words were announced at one-second intervals, in a fixed order, over 5 learning trials. Following each trial, the participant was asked to recall as many words as possible in any order. An interference list (List B) was then presented in the same fashion as list A, but over only a single trial. This list (List B) consisted of 2 shared categories from List A as well as 2 unshared categories. Following the learning trial, both free and cued recall tests of List A were performed. Free recall involved subjects announcing as many words as possible in any order. Cued recall involved subjects announcing as many words as possible after the examiner specified each category in turn. Each of these tests were performed immediately following the learning trials (short-delay) and 20 minutes following the learning trials (long-delay). The CVLT concluded with a recognition task whereby a 44-word list was read aloud. This list consisted of the 16 target words (those from list A) as well as 28 distractor words. Distractor words were specially selected to share similar categories and/or sound similar to target words. The participant was told to indicate whether each word was a target word or a distractor word as they were announced.

Inflammatory mediators of interest related to potential cognitive deficits included the proinflammatory cytokines IFN-y, IL-1, IL-2, and IL-6. Once again these cytokines were chosen due to their ability to up-regulate the enzyme IDO thereby increasing TRP degradation into metabolites such as kynurenine and quinolinic acid. The activity of IDO was assessed as well as levels of the neuroactive compounds TRP and KYN. As kynurenine is converted to kynurenic acid upon crossing the blood brain barrier and contributes to reductions in synaptic plasticity, elevated peripheral levels may be related

to cognitive impairment. Elevated levels of quinolinic acid also indicate a shift in the kynurenine pathway and has been shown to be apparent in individual's suffering from Alzheimer's disease[3].

Assessment of Neuropathic Pain

Participants were asked to complete the Neuropathic Pain Questionnaire (NPQ) at each of the 3 testing sessions, as a means of assessing self-reported neuropathic pain. The questionnaire consisted of 32 items pertaining to 3 unique categories including sensory items, affective items, and sensitivity items. Sensory items were those related to the specific type and severity of pain felt (eg. degree of burning, stabbing, throbbing, etc.), affective items referred to those related to how the pain affected the participant in daily life (eg. how irritating is your usual pain?) and sensitivity items related to how various stimuli may act to increase pain (eg. increased pain due to heat). Participants were asked to rate their pain numerically on a scale from 0-100 whereby 0 indicated the complete absence of pain and 100 indicated the worst pain imaginable. Scores from each of the 3 categories were averaged for use in statistical analysis.

Inflammatory mediators of interest related to neuropathic pain will include the proinflammatory cytokines: IL-1β, IL-2, IL-6, TNF-α and IFN-y, as well as the anti-inflammatory cytokines IL-4, IL-10, and IL-1RA. Both proinflammatory and anti-inflammatory cytokines will be examined due to the finding that painful neuropathy is typically associated with an elevation in proinflammatory cytokine while non-painful neuropathy is typically associated with an elevation in anti-inflammatory cytokines[4,5]. The enzymes COX and LOX will be examined due to their role in the production of pain-inducing pro-inflammatory eicosanoids and ability to be up-regulated by

proinflammatory cytokines. Lastly, the potent, proinflammatory eicosanoids PGE2 and LTB4 will be examined due to their role in neuropathic pain.

Assessment of Somatic Nerve Function

Action potential propagation velocity as well as M-wave amplitude were assessed by means of a nerve conduction velocity test. Both compound motor action potentials (CMAPs) and sensory nerve action potentials (SNAPs) were examined for the assessment of both motor and sensory nerves respectively. Prior to performing the test, the electrode locations were prepared by shaving the skin, removing any dead skin cells with an abrasive gel, and disinfecting the areas with rubbing alcohol. For the assessment of CMAPs, the recording electrode was placed over the muscle belly of the flexor pollicis brevis. The reference electrode was placed over the electrically neutral position of the tendon of 2nd metacarpophalangeal joint of the thumb. Lastly, a ground electrode was placed over the palm of the hand. The median nerve was then stimulated at known distance from the recording electrode at both a distal and proximal location. Stimulation intensity was determined by gradually increasing the amplitude until a maximal M-wave is achieved. A stimulation amplitude of 120% of this value was then used during testing. When electrically evoking an action potential, a standardized set of waveforms are displayed by means of an oscilloscope. A short duration spike known as the shock artifact represents the initial stimulation of the nerve and a longer duration wave known as the M-wave represents the motor response. By obtaining the time between the end of the shock artifact and initiation of the M-wave (latency), it was possible to calculate nerve conduction velocity by using the latency in conjunction with the measured distance between the stimulation and recording site. It was also possible to examine the strength of

the motor response of the flexor pollicis brevis by means of assessing the amplitude of the evoked M-wave.

When assessing SNAPs, an anti-dromic electrode configuration was utilized. Stimulation was once again performed on the median nerve at the wrist at a known distance from the recording electrode. Ring electrodes were utilized for the recording of the signal. The active recording electrode was placed just proximal to the 2nd metacarpophalangeal joint and the reference was placed just proximal to the 3rd metacarpophalangeal joint of the 4th finger. The ground electrode was placed on the dorsal surface of the hand. The amplitude of the stimulation was gradually increased until a max SNAP amplitude was achieved. The stimulation used during the testing session corresponded to 120% of the max SNAP amplitude. A 10 stimulation train was then applied. Signals were averaged and used for the determination of nerve conduction velocity and SNAP amplitude using similar techniques as discussed for CMAP analysis.

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Chapter 5

Effectiveness of Intervention: Compliance and Effect on Biomarkers

Compliance to Anti-inflammatory Diet

All participants from both the treatment and control group completed the entire 3-month duration of the study and were included in the analysis. No adverse events were reported. The treatment groups' overall compliance to the diet was assessed based on the average of the 3 diet records during the study (1 month, 2 months, and 3 months). One participant completed all three testing sessions but failed to produce the 2-month and the 3-month diet record. This participant had a dietary compliance over the first month of 92%. All other participants completed each of the required diet records and overall compliance ranged from 70-100%, with a mean compliance of 89%.

Change in Inflammatory Mediators

Changes in serum levels of inflammatory mediators are shown in Table 2. When considering a proinflammatory composite score (average of IL-2, IL-6, IL-1β, TNF-α, and IFNy), the Mann-Whitney test indicated that the change scores (3-month - baseline) were significantly different between the treatment group and the control group (U=13.0, p=0.01). The Friedman test showed that there was a statistically significant reduction in the proinflammatory composite scores in the treatment group (x^2 = 6.50, p=0.04), but no significant change in the control group (x^2=5.25, p=0.07). Post hoc analysis performed with the Wilcoxon signed-rank test showed significant reductions in the treatment group from both baseline to 1 month and baseline to 3 months (z=-2.197, p=0.03; z=-2.275, p=0.02 respectively). When analyzing each cytokine separately, the Mann-Whitney test indicated that the change scores (3-month - baseline) were significantly different between the treatment group and the control group for IFN-y (U=13.0, p=0.01.), IL-1β (U=14.0, p=0.01), and IL-2 (U=12.0, p=0.01) and showed a trend for CRP (U=27.0, p=0.10). The

Friedman test showed that in the treatment group there was a statistically significant reduction (pre to post intervention) in IFN-y (x^2=8.67, p=0.01), IL-1β (x^2=17.78, p<0.01), IL-6 (x^2=6.17, p<0.05), and a trend for CRP (x^2=4.5, p=0.10). The Friedman test showed no corresponding statistically significant reductions for any inflammatory mediator in the control group. Post-hoc analysis performed with the Wilcoxon signed-rank test showed significant reductions in the treatment group for IFN-y from baseline to 1 month and baseline to 3 months (z=-2.275, p=0.02; z=-2.510, p=0.01 respectively), as well as significant reductions in the treatment group for IL-1β from baseline to 1 month and baseline to 3 months (z=-3.059, p<0.01; z=-2.934, p<0.01 respectively), and a significant reduction in the treatment group for IL-6 from baseline to 1 month, and a trend from baseline to 3 months (z=-2.275, p=0.02; z=-1.726, p=0.08 respectively). Two-way repeated measures ANOVA were performed for the normally distributed mediators' TNF-α and PGE2 and showed trends towards group x time interactions (p=0.10; p=0.07 respectively). No significant difference was found when examining changes in the anti-inflammatory composite score (IL-4, IL-10, IL-1RA) or when examining changes in these anti-inflammatory cytokines individually.

Change in Amino Acids

Changes in serum amino acids are shown in Table 3. Two-way repeated measures ANOVA were performed for the normally distributed amino acids. There was a significant group x time interaction for the TRP/LNAA ratio (p=0.04, Cohen's d=0.90). Post hoc analysis showed a trend towards an increase in TRP/LNAA in the treatment group from baseline to 3 months (p=0.06). Post hoc analysis showed no change in the control group at any time point. KYN showed a trend towards a group x time interaction

(p=0.06, Cohen's d=0.81). A Mann-Whitney test was performed on the change scores for non-normally distributed amino acids. Change scores (3-month - baseline) were found to be significantly different between groups for the KYN/TRP ratio (U=20.0, p=0.03), BCAA (U=19.0, p=0.03), and LNAA (U=16.0, p=0.01). A Friedman's test was performed to test for significant changes among repeated measures (baseline, 1 month, 3 months) for the treatment group and control group. No significant changes were found for either the treatment group or control group.

Table #2: Change in Inflammatory Mediators

	Treatment (n=12)			Control (n=8)			2-Way ANOVA (p-value)	Mann-Whitney (p-value)	Friedman [Treat.] (p-value)
	Baseline	**1 Month**	**3 Month**	**Baseline**	**1 Month**	**3 Month**			
Pro-inflammatory Composite	20.3±34.5	13.1±23.6*	14.6±25.2*	9.8±11.6	15.4±22.3	15.7±25.3	--	<0.01	0.04
CRP (ng/ml)	4474.7±3578.9	3822.6±3749.4	2865.0±2684.9	2388.1±2928.1	3074.0±3026.4	2458.8±3678.9	--	0.10	0.10
IL-2 (pg/ml)	21.3±51.2	15.1±41.7	17.2±42.1	1.7±3.4	2.9±3.6	2.3±3.3	--	<0.01	0.23
IL-6 (pg/ml)	13.9±28.2	9.2±21.3*	9.5±19.3	9.0±10.5	13.8±21.2	13.5±21.9	--	0.13	0.049
IL-1β (pg/ml)	0.9±1.1	0.3±0.3**	0.3±0.2**	0.3±0.3	0.4±0.5	0.3±0.2	--	<0.01	<0.01
TNF-α (pg/ml)	12.5±3.6	11.8±5.5	11.2±4.1	9.8±3.9	11.3±6.7	12.9±10.3	0.10	--	--
IFN-y (pg/ml)	52.9±94.0	31.9±57.5*	35.0±68.4*	28.1±46.8	48.8±84.6	49.6±95.3	--	<0.01	0.01
Anti-inflammatory Composite	15.7±13.7	17.2±15.4	17.3±19.1	28.8±28.3	40.1±44.9	36.3±39.5	--	0.32	1.0
IL-4 (pg/ml)	7.5±20.8	12.4±23.9	16.2±38.4	19.8±37.2	37.4±83.8	23.8±46.3	--	0.54	0.63
IL-10 (pg/ml)	6.5±12.9	11.2±29.7	9.3±22.0	5.9±14.4	5.7±13.7	6.3±14.6	--	0.96	0.50
IL-1RA (pg/ml)	33.1±26.2	27.8±18.6	26.3±16.0	60.6±66.6	77.2±77.2	78.8±105.8	--	0.88	0.72
PGE2 (pg/ml)	496.5±452.7	636.4±544.9	353.0±357.5	605.1±491.2	605.6±504.6	661.7±503.7	0.07	--	--
LTB4 (pg/ml)	127.8±181.8	119.8±194.9	77.0±73.8	121.3±172.3	87.6±59.3	145.1±179.8	--	0.70	0.78

All results are shown as mean ± SD. P-values correspond to group x time interactions, Mann-Whitney change scores, and Friedman scores for treatment group respectively.
Proinflammatory composite consists of a composite score averaging IL-2, IL-6, IL-1β, TNF-α, and IFN-y
Anti-inflammatory composite consists of a composite score averaging IL-4, IL-10, and IL1RA
**Significantly different from baseline with P value <0.05; **Significantly different from baseline with P value <.01*

Table #3: Change in Amino Acids

	Treatment (n=12)			Control (n=8)			2-Way ANOVA (p-value)	Mann-Whitney (p-value)
	Baseline	**1 Month**	**3 Month**	**Baseline**	**1 Month**	**3 Month**		
TRP	89.2±19.4	87.6±28.8	90.7±26.7	107.8±28.1	105.9±20.3	102.6±14.4	0.86	--
TYR	48.2±17.7	50.5±15.1	41.5±15.2	59.4±17.6	53.7±15.7	62.2±18.3	0.11	--
PHE	36.6±13.6	36.8±13.6	33.6±14.8	42.0±16.6	40.8±18.2	46.2±23.4	--	0.17
BCAA	700.2±462.4	651.3±351.9	467.9±198.5	449.7±105.5	472.2±112.1	534.3±159.2	--	0.03
LNAA	785.0±468.2	738.6±352.3	543.1±217.3	551.2±108.7	566.8±123.8	642.7±190.3	--	0.01
TRP/LNAA	146.9±71.3	133.6±52.3	188.7±90.4	206.3±79.6	194.0±51.2	171.2±49.8	0.04	--
KYN	2.3±0.7	2.3±0.6	2.1±0.4	1.7±0.4	2.1±0.3	2.1±0.4	0.06	--
PHE/TYR	786.8±232.2	738.1±229.5	809.5±180.4	746.6±247.1	751.08±219.1	732.78±267.2	0.44	--
KYN/TRP	27.0±11.6	28.5±10.9	24.0±6.6	16.8±4.9	20.3±4.7	20.6±6.0	--	0.03

All amino acids are displayed in µmol/L
All results are shown as mean ± SD. P-values correspond to group x time interactions and Mann-Whitney test on change scores.
TRP: tryptophan; TYR: tyrosine; PHE: phenylalanine; BCAA: branch chain amino acid; LNAA: large neutral amino acids; KYN: kynurenine.

Chapter 6

Manuscript 1

Targeting inflammation to influence mood following spinal cord injury: A randomized clinical trial

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ABSTRACT

Background: The purpose of the present study was to examine the efficacy of targeting inflammation as a means of improving mood following spinal cord injury (SCI) and explore the potential mechanisms of action.

Methods: The study was a randomized, parallel-group, controlled, clinical trial (NCT02099890) whereby 20 participants with varying levels and severities of SCI were randomized (3:2) to either the treatment group, consisting of a 12-week anti-inflammatory diet, or control group. Outcome measures were assessed at baseline, 1 month, and 3 months and consisted of CES-D scores of depression, markers of inflammation as assessed by various pro and anti-inflammatory cytokines, and several amino acids related to depression.

Results: A significant group x time interaction was found for CES-D (center for epidemiological studies depression scale) score ($p=0.01$) and the TRP/LNAA (tryptophan/large neutral amino acid) ratio ($p=0.04$). A Mann-Whitney test indicated change scores (3 month-baseline) were significantly different between the treatment group and control group for a composite score of pro-inflammatory mediators ($U=13.0$, $p=0.01$), IFN-y ($U=13.0$, $p=0.01$.), IL-1β ($U=14.0$, $p=0.01$), and IL-2 ($U=12.0$, $p=0.01$) and showed a trend for CRP ($U=27.0$, $p=0.10$). A Friedman test showed significant reductions in the treatment group for pro-inflammatory composite scores ($x^2 = 6.50$, $p=0.04$), IFN-y ($x^2=8.67$, $p=0.01$), IL-1β ($x^2=17.78$, $p<0.01$), IL-6 ($x^2=6.17$, $p<0.05$), and a trend for CRP ($x^2=4.5$, $p=0.10$), while the control had no significant changes for any inflammatory mediators. Pearson's r correlation showed significance between the ΔIL-1β and both the ΔCES-D score ($r=.740$, $p<.01$) and the ΔKYN/TRP (kynurenine/tryptophan) ratio ($r=.536$, $p=0.02$). The ΔKYN/TRP ratio was also significantly correlated with the ΔCES-D score ($r=.586$, $p=0.01$). Mediation analysis showed that the relationship between the ΔKYN/TRP ratio and the ΔCES-D score was mediated by the ΔIL-1β. Subgroup analysis showed participants with high CES-D scores had significantly higher concentrations of IL- 1β and all correlations were maintained or strengthened within this subgroup.

Conclusions: Overall, the results demonstrated the effectiveness of targeting inflammation as a means of improving mood in SCI, with potential mechanisms relating to the reduction in IL-1β and improvements in levels of neuroactive compounds related to the kynurenine pathway. Due to the limited sample size, results should be interpreted with caution, however, they are worthy of further examination due to the potential impact of inflammation on depression.

Trial Registration: ClinicalTrials.gov ID: NCT02099890

Keywords: Depression; Mood; Spinal Cord Injury; Inflammation; IL-1β; Anti-inflammatory Diet

Background

Individuals with major depressive disorder (MDD) are commonly reported to demonstrate immune dysfunction in the form of chronic inflammation and likewise, individuals with chronic inflammatory disorders are more prone to MDD.[1–3] Such a state has been proposed to be a contributing factor to symptoms of depression due to the complex bidirectional communicatory pathways between various systems of the body. In this respect, chronic inflammation may impact the endocrine and nervous systems causing respective imbalances in critical hormones and neuroactive compounds which may ultimately influence behavior and contribute to depressive symptoms.[4]

Various proinflammatory mediators possess the ability to influence the function of transporters, enzymes, and receptors both in the periphery and within the brain. Certain proinflammatory cytokines are able to travel from the periphery to the brain via both active and passive mechanisms.[5,6] Once in the brain, proinflammatory cytokines such as interleukin-1β (IL-1β) and tumour necrosis factor-α (TNF-α) can alter extra-cellular concentrations of serotonin (5-HT) by up-regulating corresponding transporters (SERT).[7–9] In this way, such proinflammatory cytokines have the ability to directly reduce 5-HT levels within the brain which are associated with symptoms of depression.

Peripherally, proinflammatory cytokines can influence the regulation of enzymes from critical metabolic pathways and induce imbalances in key mood altering neuroactive compounds. The kynurenine pathway is one such pathway which must be strictly regulated due to its role as the primary route for tryptophan (TRP) degradation. Approximately 95% of this critical 5-HT precursor is metabolized along this pathway meaning a cytokine-induced upregulation of TRP degradation may ultimately result in

the reduction in 5-HT levels within the brain.[10,11] In the same respect, the increased degradation of TRP results in elevated levels of TRP metabolites. Certain TRP metabolites such as kynurenine (KYN) are BBB (blood brain barrier) transportable and have also been shown to contribute to symptoms of depression when at elevated concentrations within the brain (see Figure 1a and b).

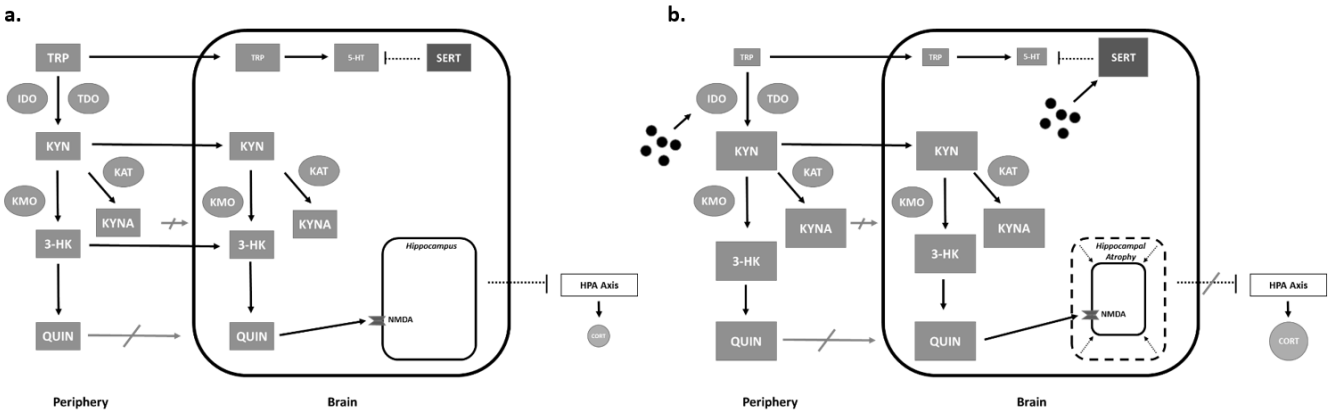

Figure #1: Tryptophan Metabolism along the Kynurenine Pathway

Figure 1a depicts the kynurenine pathway within the periphery and brain under healthy conditions. Proper enzyme regulation results in a healthy balance of TRP and TRP metabolites. As a result, adequate levels of TRP are available for 5-HT synthesis and properly regulated SERT proteins ensure extracellular concentrations of 5-HT are not depleted. Healthy levels of TRP metabolites within the brain (eg. QUIN) ensure normal activation of NMDA receptors thereby maintaining healthy hippocampal function and proper regulation of the HPA axis.

Figure 1b depicts the kynurenine pathway within the periphery and brain in a state of chronic inflammation. Elevated levels of proinflammatory cytokines cause up-regulation of enzymes such as IDO thereby resulting in an increased breakdown of TRP and production of TRP metabolites. As a result, reduced levels of TRP are available for the synthesis of 5-HT. Proinflammatory cytokines also up-regulate SERT proteins causing further depletion of extracellular 5-HT. Elevated levels of TRP metabolites within the brain such as QUIN (potent NMDA agonist) result in over-activation of NMDA receptors, neuronal damage, and potential atrophy. This may also lead to a loss of inhibition of the HPA axis and excessive production of stress hormones.

A low-grade chronic inflammatory state is very commonly reported following spinal cord injury (SCI) as characterized by elevated levels of circulating pro-inflammatory mediators.[12–16] This state of immune dysfunction can be attributed to a variety of factors ranging from the loss of autonomic innervation of lymphoid organs, endocrine dysfunction, metabolic disorders, and a heightened risk for secondary health complications.[17] This population is also far more prone to depression with rates reaching up to 5 times (20-40%) that of healthy, able-bodied individuals.[18,19] Suicide rates are also far more prevalent among this population with rates ranging from 3-5 greater than healthy able-bodied individuals.[20,21] Provided that traditional pharmaceutical treatments for depression, such as selective serotonin reuptake inhibitors (SSRI's), have commonly reported side effects,[22] prove ineffective in approximately 30% of patients,[23] and correspond to high rates of relapse,[24] alternative treatments are needed.

The link between inflammation and depression may provide the opportunity to treat the latter by targeting the immune system via anti-inflammatory strategies. As there is a need for drastic changes in dietary intake following paralysis, and these changes are typically not met, the eating habits of individuals with SCI commonly contribute to a state of chronic inflammation. This provides the opportunity to reduce inflammation via the implementation of a strict anti-inflammatory diet for the purposes of assessing the influence of reduced inflammatory mediators on mood following SCI. It was hypothesized that reducing inflammation would lead to corresponding changes in neuroactive compounds and improvements in mood and depressive symptoms.

Methods

**See Chapter 4 - Overview of Study Design for methodologies regarding participants, randomization, and anti-inflammatory diet*

Measurement of serum inflammatory markers and amino acids:

Blood draws (20ml) were taken from the antecubital vein of each participant at 1pm at each of the 3 testing sessions (baseline, 1 month, and 3 months). Following extraction, the whole blood was allowed to clot for 30 minutes followed by centrifugation at 1000xg for 15 minutes. Serum was extracted and immediately stored at -80°C until later analysis. Inflammatory mediators of interest included the pro-inflammatory cytokines IL-2, IL-1β, IL-6, TNF-α, IFN-y, the acute phase protein CRP, as well as the anti-inflammatory cytokines IL-4, IL-10, and IL-1RA. Amino acids of interest included tryptophan, phenylalanine, tyrosine, and branched-chain amino acids (valine, leucine, isoleucine) for the assessment of the TRP/LNAA ratio. Phenylalanine and tyrosine were also quantified for the assessment of the PHE/TYR ratio which provides an indication of phenylalanine (4)-hydroxylase activity. Chronic inflammation has been shown to impair the activity this enzyme thereby causing increases in PHE and an increase in the PHE/TYR, which provides some indication of the inflammatory state.[25] Kynurenine levels were also analyzed for the assessment of the KYN/TRP ratio. Analysis of pro and anti-inflammatory cytokines was performed in triplicate via the Magpix Multiplex system (EMD Millipore, MA, U.S.A) and analyzed using Luminex software. CRP was analyzed in triplicate and quantified via enzyme-linked immunosorbent assay (R&D systems, Minneapolis, U.S.A.). TRP, Phe, TYR, BCAA, and KYN were analyzed in triplicate and quanitified via enzyme-linked immunosorbent assay (Immunodiagnostik, Bensheim, Germany; Labor Diagnostika, Nordhorn, Germany).

Assessment of mood:

Participants completed the Center for Epidemiological Studies Depression Scale (CES-D) at each of the 3 testing sessions, as means of assessing symptoms of depression. The questionnaire consisted of 20 items related to depression and participants were asked to rate how often they experienced each item over the previous 7-day period. Ratings were based on a 4-point scale including, 'rarely or none of the time' (less than 1 day), 'some or a little of the time' (1-2 days), 'occasionally or a moderate amount of the time' (3-4 days), or 'most or all of the time' (5-7 days). Points for each item ranged from 0-3 depending on frequency and each item was summed for a total score ranging from 0-60 with higher scores indicating the presence of more symptomatology. A score of 16 points or greater is considered depressed.

Statistical analysis:

Two-way (group x time) repeated measures ANOVA were performed to investigate possible changes in CES-D scores across 3 testing sessions. Two-way repeated measures ANOVA were also performed for the proinflammatory cytokine TNF-α and the amino acids TRP, TYR, KYN, and the TRP/LNAA ratio. Post hoc analyses were used as needed to compare means when significant group x time interactions were found. These data are expressed as means ± standard deviations. As the remaining inflammatory mediators and amino acids were not normally distributed, non-parametric analyses were performed. A Friedman's test of differences among repeated measures (baseline, 1 month, 3 month) for the treatment group and control was performed. If the Friedman's test resulted in a significant value, a Wilcoxon signed-rank test was then performed to provide specific information regarding which time points were significantly

different from one another. Finally, A Mann-Whitney test was performed on change scores (3-month - baseline) between groups to establish if the change experienced in inflammatory mediators and amino acids significantly differed between groups. These data are expressed as means ± standard deviations. Correlations between changes in inflammatory mediators, amino acids, and CES-D scores were assessed by means of Pearson's r correlation. A mediation analysis was performed to assess whether the relationship between the change in the KYN/TRP ratio and the change in CES-D score was mediated by the change in IL-1β. This analysis consisted of four steps of testing: (1) the association of the independent variable (ΔKYN/TRP ratio) with the outcome variable (ΔCES-D score), (2) the association of the independent variable with the mediator variable (ΔIL-1β), (3) the association of the mediator variable with the outcome variable after controlling for independent variable, and (4) whether the effect of the independent variable on the dependent variable was reduced when the mediator was included in the model. Differences in IL-1β concentrations for subgroup analysis were assessed using the Student's t-test. Statistical significance was set at $p \leq 0.05$ for all tests.

Results

All participants from both the treatment and control group completed the entire 3-month duration of the study and were included in the analysis. No adverse events were reported. The participants' overall compliance to the diet was assessed based on the average of the 3 diet records during the study (1 month, 2 months, and 3 months). One participant completed all three testing sessions but failed to produce the 2-month and the 3-month diet record. This participant had a dietary compliance over the first month of 92%. All other participants completed each of the required diet records and overall

compliance ranged from 70-100%, with a mean compliance of 89%. A detailed analysis regarding specific diet adherence data will be presented elsewhere.

Change in CES-D scores:

Change in CES-D scores are shown in Table 1. There was a significant group x time interaction for CES-D scores (p=0.01, Cohen's d=1.07). Post hoc analysis showed a significant reduction in CES-D scores in the treatment group from both baseline to 1 month, as well as from baseline to 3 months (p=0.01 and p<0.01 respectively). Post hoc analysis showed a significant reduction in CES-D score in the control group from baseline to 1 month (p=0.03) but no significant change from baseline to 3 months (p=.74).

Table 1: CES-D Scores:

	Treatment (n=12)			Control (n=8)		
	Baseline	**1 Month**	**3 Month**	**Baseline**	**1 Month**	**3 Month**
CES-D Score	14.5±10.7	6.8±5.6**	6.5±5.0**	13.9±12.0	11.3±10.3*	14.6±13.6

Change in Serum Amino Acids:

Changes in serum amino acids are shown in Table 3 of Chapter 5. Two-way repeated measures ANOVA were performed for the normally distributed amino acids. There was a significant group x time interaction for the TRP/LNAA ratio (p=0.04, Cohen's d=0.90). Post hoc analysis showed a trend towards a reduction in TRP/LNAA in the treatment group from baseline to 3 months (p=0.06) and no change in the control group at any time point. No significant group x time interaction was found for the PHE/TYR ratio (p=.435, Cohen's d=0.43). KYN showed a trend towards a group x time

interaction (p=0.06, Cohen's d=0.81). A Mann-Whitney test was performed on the change scores for non-normally distributed amino acids. Change scores (3-month - baseline) for the KYN/TRP ratio were shown to be significantly different between the treatment and control groups (U=20.0, p=0.03). A Friedman's test was performed to test for significant changes among repeated measures (baseline, 1 month, 3 months) for the treatment group and control group. No significant change in the KYN/TRP ratio was found in the treatment (x^2=2.09, p=0.35) or control (x^2=4.75, p=0.09) group.

Change in Inflammatory Mediators:

Changes in serum levels of inflammatory mediators are shown in Table 2 of Chapter 5. When considering a proinflammatory composite score (average of IL-2, IL-6, IL-1β, TNF-α, and IFNy), the Mann-Whitney test indicated that the change scores (3-month - baseline) were significantly different between the treatment group and the control group (U=13.0, p=0.01). The Friedman test showed that there was a statistically significant reduction in the proinflammatory composite scores in the treatment group (x^2 = 6.50, p=0.04), but no significant change in the control group (x^2=5.25, p=0.07). Post hoc analysis performed with the Wilcoxon signed-rank test showed significant reductions in the treatment group from both baseline to 1 month and baseline to 3 months (z=-2.197, p=0.03; z=-2.275, p=0.02 respectively). When analyzing each cytokine separately, the Mann-Whitney test indicated that the change scores (3-month - baseline) were significantly different between the treatment group and the control group for IFN-y (U=13.0, p=0.01.), IL-1β (U=14.0, p=0.01), and IL-2 (U=12.0, p=0.01) and showed a trend for CRP (U=27.0, p=0.10). The Friedman test showed that in the treatment group there was a statistically significant reduction in IFN-y (x^2=8.67, p=0.01), IL-1β

(x^2=17.78, p<0.01), IL-6 (x^2=6.17, p<0.05), and a trend for CRP (x^2=4.5, p=0.10). The Friedman test showed no statistically significant reductions for any inflammatory mediator in the control group. Post-hoc analysis performed with the Wilcoxon signed-rank test showed significant reductions in the treatment group for IFN-y from baseline to 1 month and baseline to 3 months (z=-2.275, p=0.02; z=-2.510, p=0.01 respectively), as well as significant reductions in the treatment group for IL-1β from baseline to 1 month and baseline to 3 months (z=-3.059, p<0.01; z=-2.934, p<0.01 respectively), and a significant reduction in the treatment group for IL-6 from baseline to 1 month, and a trend from baseline to 3 months (z=-2.275, p=0.02; z=-1.726, p=0.08 respectively). Two-way repeated measures ANOVA were performed for the normally distributed mediator, TNF-α which showed a trend towards a group x time interaction (p=0.10, Cohen's d=0.12)

Correlational Analysis:

Pearson's r correlation coefficients for amino acid, cytokine, and CES-D data are shown in Table 2. The change in IL-1β was significantly correlated with both the change in CES-D score (r=.740, p<0.01) and the change in the KYN/TRP ratio (r=.536, p=0.02). The change in the KYN/TRP ratio was also significantly correlated with the change in CES-D score (r=.586, p=0.01). The relationship between the change in TRP/LNAA ratio and the change in CES-D score did not reach statistical significance (r=-0.378, p=0.10), whereas the relationship between the change in the TRP metabolite KYN and the change in CES-D score did reach statistical significance (r=.705, p<0.01).

Table 2. IL-1β, Amino acid, and CES-D Correlations

All Participants [n=20]				
	Δ*CES-D*	**Δ*IL-1B***	**Δ*TRP/LNAA***	**Δ*KYN/TRP***
Δ*CES-D*	--			
Δ*IL-1B*	.740**	--		
Δ*TRP/LNAA*	-.378	.369	--	
Δ*KYN/TRP*	.586**	.536*	.345	--
Δ*KYN*	.705**	.657**	.114	.730**
Subgroup (CES>16) [n=8]				
	Δ*CES-D*	**Δ*IL-1B***	**Δ*TRP/LNAA***	**Δ*KYN/TRP***
Δ*CES-D*	--			
Δ*IL-1B*	.738*	--		
Δ*TRP/LNAA*	.569	.363	--	
Δ*KYN/TRP*	.937**	.792*	.436	--
Δ*KYN*	.800**	.825**	.192	.823**

*p≤.05; **p≤ *0.01*

Mediation Analysis:

A mediation analysis was performed in order to test whether the change in IL-1β mediated the observed relationship between the change in KYN/TRP and the change in CES-D scores. In the first step of the mediation model, the association of the independent variable with the outcome variable was assessed. This regression of the change in the KYN/TRP ratio on the change in CES-D scores (ignoring the change in IL-1β) was significant, b=.586, t(18)=3.07, p=0.01. In step two the association of the independent variable with the mediator variable was assessed. It was shown that the regression of the change in the KYN/TRP ratio on the mediator (change in IL-1 β) was also significant, b=0.06, t(18)=2.70, p=0.02. In step 3 of the mediation process the association of the mediator variable with the outcome variable after controlling for the independent variable was assessed. The regression of the change in IL-1β on the change in CES-D score

(controlling for the change in the KYN/TRP ratio) was significant b=5.34, t(17)=3.30, p<0.01. In the last step we assessed whether the effect of the independent variable on the dependent variable was reduced when the mediator was included in the model. When controlling for the change in IL-1β, the change in the KYN/TRP ratio was not a significant predictor of the change in CES-D score, b=0.25, t(17)=1.45, p=0.16. A Sobel test was conducted and found full mediation in the model (z=2.04, p=0.04). Therefore, it was found that the change in IL-1β fully mediated the relationship between the change in the KYN/TRP ratio, and the change in CES-D scores.

Subgroup Analysis:

A subgroup analysis was performed examining participants with CES-D scores greater than 16 (indicating depression) from both the treatment group (n=5) and control group (n=3). It was found that participants with CES-D scores indicating depression had concentrations of IL-1β which were 73% higher compared to those with lower (<16) CES-D scores (p=0.05). Pearson's r correlation coefficients for amino acid, cytokine, and CES-D data are shown in Table 4. The significant relationship between change in IL-1β and the change in CES-D remained in this subgroup (r=.738, p=0.04) and the relationship between the change in IL-1β and the change in the KYN/TRP ratio became even stronger (r=.792, p=0.02). The change in the KYN/TRP ratio and the change in CES-D was also very strongly correlated in this subgroup (r=.937, p<0.01). Once again the change in the TRP/LNAA and the change in CES-D were not found to be significantly correlated (r=.569, p=0.11) while the change in KYN was strongly correlated with the change in CES-D score (r=.800, p=0.01).

Discussion

The present study successfully obtained reductions in inflammatory mediators, modifications in neuroactive compounds, and improvements in mood in individuals with SCI by means of dietary alterations. As evidence suggests inflammation contributes to depression, and SCI is typically characterized by a state of chronic inflammation as well as a high prevalence of depression, it was hypothesized that reducing levels of inflammation in this population would result in both molecular changes and corresponding improvements in mood.

Mood was significantly improved in the treatment group as demonstrated by the significant reduction in CES-D scores. When assessing potential mechanisms for such changes, a significant group x time interaction for the TRP/LNAA ratio was found and post hoc analysis revealed trends towards an increase in the treatment group at 1 month and 3 months following baseline. TRP is a critical precursor for 5-HT synthesis in the brain and competes for transportation across the BBB against other LNAA via common transport mechanisms. As such, an increase in the TRP/LNAA ratio signifies heightened TRP availability and would be expected to relate to improvements mood. However, the relationship between the change in the TRP/LNAA ratio and the change in CES-D scores did not reach statistical significance ($p=0.10$).

A significant group x time interaction was found for both IFN-y and IL-1β and post hoc analysis showed trends towards reduction for IFN-y at 1 month and 3 months, and a significant reduction for IL-1β at 1 month and trend towards reduction at 3 months. Trends toward group x time interactions were also found for IL-6, TNF-α, and IL-1RA. Of these changes, only the reduction in IL-1β was shown to significantly correlate with

the reduction in CES-D score. As IL-1β is capable of gaining access to the brain via leaky sites at the circumventricular organs or by crossing the BBB or via specialized active transporters it may be possible for it to impose a direct influence.[5,6] Once in the brain, IL-1β has been shown to activate 5-HT transporters (SERT), thereby stimulating the re-uptake of 5-HT from the synaptic cleft, leading to its functional depletion.[7-9] Although it was not possible to directly assess SERT or 5-HT levels within the brain, it is possible for IL-1β to have contributed to changes in mood by means of such direct mechanisms.

The change in IL-1β was also significantly correlated with the change in the KYN/TRP ratio. The KYN/TRP ratio can represent the activity of either indoleamine 2,3-dioxygenase (IDO) or tryptophan 2,3 dioxygenase (TDO). Both are critical enzymes of the kynurenine pathway responsible for the degradation of TRP into KYN. TDO is however, mainly expressed in the liver and not regulated by the immune system.[26] IDO is an immunoregulated enzyme and elevations in the KYN/TRP ratio have been previously shown to be significantly correlated with increases in IFNy activity[27] as well as elevations in neopterin; a molecule synthesized by macrophages upon stimulation by IFNy, and indicator of a pro-inflammatory status.[28] This strongly suggests that IDO is the main enzymatic contributor to changes in the KYN/TRP ratio in response to changes in inflammation. Additional pro-inflammatory cytokines, including IL-1β, have been shown to up-regulate IDO activity, thereby increasing the rate of TRP metabolism and production of TRP metabolites.[29] This selective TRP metabolism can lead to reductions in the TRP/LNAA ratio thereby limiting TRP availability for the synthesis of 5-HT. Additionally, the excessive breakdown of TRP results in the increased production of TRP metabolites which have also been shown to play an important role in factors related to

depression. As the change in the KYN/TRP ratio was also significantly correlated with the change in CES-D scores it may be possible that IL-1β influenced depression via such IDO-related mechanisms. Based on these findings, a mediation analysis was performed to further address the extent to which the link between change in the KYN/TRP ratio and change in the CES-D scores were mediated by change in the IL-1β. The analysis suggested that the relationship between change in the KYN/TRP and change in the CES-D was fully mediated by change in the IL-1β.

In addition to the potential reduction in TRP availability as shown by the TRP/LNAA ratio, a cytokine-induced up-regulation of IDO activity may result in elevations in TRP metabolites. Several TRP metabolites, including KYN, are BBB transportable and can be neurotoxic when at elevated concentrations within the brain. As in the periphery, once in the brain KYN can be further metabolized along the kynurenine pathway into other metabolites which have been shown to influence depression. One such metabolite, quinolinic acid (QUIN), acts as a potent agonist of the N-methyl-D-aspartate (NMDA) receptor which are densely populated on the hippocampus and play a critical role in synaptic plasticity. Elevated concentrations of QUIN may result in NMDA over-activity resulting in an increased calcium influx leading to corresponding neuronal damage and potential atrophy.[30] Smaller hippocampal volumes in individuals with MDD have been commonly reported.[31-34] As the hippocampus plays an important role concerning the inhibition of HPA axis, hippocampal atrophy may result in an upregulated HPA axis and excessive glucocorticoid production from the adrenal gland. Hyperactivity of the HPA axis and elevated levels of stress hormones have also been commonly reported in individuals with MDD (see Figure 1).[35,36]

Both the reduction in the TRP/LNAA ratio and increase in TRP metabolites have the potential to influence depression via the aforementioned mechanisms. However, the fact that the change in KYN was related to the change in CES-D scores, (while the change in TRP/LNAA was not) may suggest that the production of TRP metabolites had a larger impact on such mood changes. Similar findings have been demonstrated by Sublette et al. 2011, whereby levels of KYN have been shown to be elevated in suicide attempters with MDD and correlated with attempt status, while TRP was not related. Further, Sublette et al. found a correlation between the cytokine activation marker neopterin and the KYN/TRP ratio suggesting that KYN production may have been influenced by inflammation.[37]

Additional subgroup analysis on those with baseline CES-D scores greater than 16 (suggesting depression) further implicated a role for IL-1β in depression as levels were shown to be 73% higher than in those with CES-D scores indicating a lack of depression. Further, all relationships between IL-1β, the KYN/TRP ratio and CES-D scores were maintained or strengthened in this subgroup including a very strong relationship between the change in the KYN/TRP ratio and the change in CES-D scores. Despite the small sample size, it also worth noting that for the five participants in the treatment group who began with scores indicating depression, all five reached scores below 16 within the first month of the intervention.

The above mechanisms may explain the link between the dietary-induced reductions in inflammation and the improvements in mood as assessed by CES-D scores. SCI is associated with substantial alterations in nutrient requirements and energy demands as well as an increased risk for numerous metabolic disorders and acute

infections. Appropriate dietary alterations are therefore a suitable method of reducing inflammation and have a number of established mechanisms of action ranging from altered gene transcription, changes in cell membrane composition, improved enzyme regulation, as well as improvements in metabolic heath and body composition.[38]

Common pharmaceutical treatment modalities for depression, such as selective serotonin reuptake inhibitors (SSRI's), are popular due to their ability to relieve symptoms in a relatively short period of time by acting on SERT proteins to help increase extracellular 5-HT levels. SSRI use is, however, associated with a number of side effects[22], and only provide transient relief of symptoms as they do not target the etiological basis of the disorder. SSRI's have also been shown to be ineffective in approximately 30% of patients[23] of whom a particularly elevated inflammatory state is typically reported.[39-40] Additionally, of those who do respond to treatment, an estimated 20-80% will relapse within the first 1-5 years following initial treatment.[24] As such, there is a clear need for a more sustainable, long-term treatment, free from the many side-effects that coincide with prolonged use of traditional pharmaceutical treatments.

Whether anti-inflammatory strategies could be used as a stand-alone treatment or would need to be used in conjunction with other interventions has yet to be determined. Future, long-term studies with larger sample sizes, and participants with various severities of depression will be needed. Several potential limitations of the current study should be noted. First, the study was only single blinded. While the examiner was blinded to group allocation during all blood analysis, participants were aware of their group assignment. Although placebo supplements could have been provided to the control group, it was not possible to adequately blind participants to all aspects of the diet. The

treatment group underwent a highly restrictive diet while the control group was free to consume unhealthy foods making distinction between groups quite obvious. Second, it is not possible to elucidate the specific mechanisms related to the reductions in inflammation, nor is it possible to discern which aspects of the dietary intervention may have had the strongest effects. It will be necessary for future studies to examine aspects such as transcription factor activity and membrane composition in order to truly elucidate the means by which such interventions act to reduce inflammation and improve symptoms of neuropathic pain. In terms of generalizability our sample was quite representative of the SCI population in Canada, in terms of age, level, and severity of injury[41] however, the results may not necessarily be generalizable to all individuals with SCI with varying levels of inflammation and severities of depression (however, it is of interest that all participants with scores indicating depression improved to a score indicating a lack of depression in the current study). Finally, it is worth noting that utilizing an intervention which targets inflammation by such means as diet or exercise requires a commitment to a major lifestyle modification and may not provide the same immediacy of effects as traditional pharmaceuticals. Still, the current study showed strong compliance to the diet and achieved significant reductions in CES-D scores after only 1 month. Further, given the lack of side effects, and additional health benefits that coincide with healthy eating, such interventions may be a viable mode of treatment for this population and are worthy of further examination.

Conclusion

The present study demonstrated that it was possible to improve mood in individuals with SCI by means of reducing inflammation. Such improvements may relate

to positive changes in neuroactive compounds of the kynurenine pathway mediated by the significant reduction achieved in the proinflammatory cytokine IL-1β. These results suggest a potential role for anti-inflammatory interventions in the treatment of depression in spinal cord injured individuals. Due to the limited sample size of the present study, the results should be interpreted with caution. However, this influence is worthy of further examination in future larger scale studies, as it may help to reduce the reliance on traditional pharmaceuticals by complementing current treatments or by providing a safe and sustainable treatment alternative.

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Conflict of Interest:

The authors declare no conflict of interest.

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Chapter 7

Manuscript 2

Targeting inflammation to influence cognitive function following spinal cord injury: A Randomized Clinical Trial

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Submitted to: Spinal Cord

Abstract

Study Design: This study was a randomized, parallel-group, controlled clinical trial (NCT02099890).

Objectives: The purpose of this study was to examine the efficacy of targeting inflammation as a means of improving cognitive function in individuals with spinal cord injury.

Setting: Participants were recruited from the Niagara region of Ontario Canada

Methods: Indices of memory and verbal learning were assessed by means of the California Verbal Learning Test (CVLT). Inflammation and concentrations of neuroactive compounds related to the kynurenine pathway were assessed via a number of pro and anti-inflammatory cytokines, as well as tryptophan, kynurenine, and several large neutral amino acids. All assessments were performed at baseline as well as at 1-month and 3-months during a 3-month anti-inflammatory intervention.

Results: Despite a reduction in inflammation, only intrusions were found to significantly improve in the treatment group ($p=0.05$). All other CVLT measures including list A, trial 1 ($p=0.48$), learning slope ($p=0.46$), long delay free recall ($p=0.83$), and repetitions ($p=0.07$) showed no significant group x time interaction.

Conclusion: It may be possible that the reduction in inflammation achieved in the current study was insufficient to induce substantial changes in most indices of verbal learning and memory. Alternatively, as these participants likely underwent years of previous chronic inflammation, underlying hippocampal damage may have negated potential improvements induced by acute reductions in inflammation. The isolated significant improvement in intrusions is difficult to explain, but may relate to the role of the frontal lobe in source memory and the ability to avoid incorrect responses.

Trial Registration: ClinicalTrials.gov ID: NCT02099890

Sponsorship: This study was funded by the Ontario Neurotrauma Foundation

Keywords

Cognition; Spinal Cord Injury; Inflammation; CVLT

Introduction

An estimated 10-60% of individuals with spinal cord injury (SCI) demonstrate some degree of cognitive impairment[1–4]. This dysfunction includes memory impairment[4–6], and deficits in verbal learning[4–6], which would suggest a role for the hippocampus[7]. Cognitive deficits following SCI are often attributed to concomitant traumatic brain injury, and more recently, chronic hypotension[8], however, there is evidence to suggest that the chronic inflammation typically demonstrated following SCI[9,10] may also contribute to such deficits. Populations with severe cognitive impairment, such as those with Alzheimer's disease, have been consistently shown to demonstrate high levels of inflammation[11] and this elevated inflammatory state may indirectly influence hippocampal function.[12]

Certain pro-inflammatory mediators such as IFN-y[13,14], IL-1β[14], and TNF-α[13,14] have been shown to possess the ability to up-regulate indoleamine 2,3 dioxygenase (IDO); a key enzyme of a critical metabolic pathway known as the kynurenine pathway. This enzyme plays a vital role in the metabolism of tryptophan (TRP) and the corresponding production of TRP metabolites such as kynurenine (KYN).[12] Chronically elevated levels of proinflammatory mediators may therefore result in the chronic upregulation of IDO, resulting in excessive TRP breakdown and the overproduction of TRP metabolites such as KYN. As KYN is a blood brain barrier (BBB) transportable metabolite, it can move from the periphery and influence concentrations within the brain. Once in the brain, KYN can be further metabolized into non-BBB transportable metabolites such quinolinic acid (QUIN) and kynurenic acid (KYNA). These metabolites are produced via two distinct branches of the kynurenine pathway. The kynurenine-

nicotinamide adenine dinucleotide (KYN-NAD) branch involves the enzyme kynurenine monooxygenase (KMO) and is responsible for converting KYN to 3-hydroxykynurenine (3-HK) and QUIN. A second branch, known as the kynurenine-kynurenic acid (KYN-KYNA) branch, involves the enzyme kynurenine aminotransferase (KAT) and is responsible for the conversion of KYN to KYNA[15] (see Figure 1). The enzyme KMO has been shown to be far less active within the brain than in the periphery, and as such, becomes rapidly saturated by elevated levels of KYN. It is therefore likely, that in the presence of high KYN levels within the brain, a shift towards the KYN-KYNA branch would occur resulting in the increased production of KYNA[16]. KYNA is a neuroactive compound which acts as an antagonist of two receptors which have been found to be densely populated on the hippocampus including the alpha-7-nicotinic acetylcholine (α7nACh) receptor and (to a lesser extent) the N-methyl-D-aspartate (NMDA) receptor. Each of these receptors play a critical role in synaptic plasticity associated with learning and memory[17]. The inhibition of α7nACh receptors by KYNA has been shown to result in the reduced release of neurotransmitters such as glutamate, acetylcholine, and dopamine; each which plays a critical role in cognitive processes.[18–20] Therefore, by influencing neuroactive compounds of the kynurenine pathway, it may be possible for chronically elevated levels of inflammation to indirectly influence cognitive processes.

Evidence of this relationship has been demonstrated in animal models whereby elevations in KYNA within the brain have been shown to induce cognitive deficits whether induced indirectly via intraperitoneal administration of KYN[21] or via direct intracerebroventricular KYNA infusion[22]. Further, KAT II knockout mice, who lack the enzyme responsible for producing KYNA have been shown to have 66% lower

extracellular concentrations of KYNA as well as superior cognitive performance[17]. In humans, the administration of the non-competitive NMDA glutamate receptor antagonist, ketamine, has been shown to result in a reduction in verbal declarative memory[23]. Individuals with Alzheimer's disease have also been shown to exhibit reduced concentrations of TRP and heightened concentrations of QUIN in the periphery[24]. Further, elevated concentrations of the potent IDO activator TNF-α, has been demonstrated in this population, and the administration of the TNF- α antagonist, etanercept, has been shown to result in improved cognitive scores[11,25].

Despite the evidence pertaining to the mechanisms behind such inflammatory-induced cognitive deficits and changes in outcome in both animal models and humans, no study has attempted to define the inflammatory etiology of cognitive deficits in individuals with SCI. The purpose of the present study was therefore to assess whether reducing levels of inflammation in individuals with SCI would be sufficient to induce improvements in memory and verbal learning. It was hypothesized that due to the role chronic inflammation plays in the regulation of the kynurenine pathway, reductions in inflammation would result in improved scores memory and verbal learning.

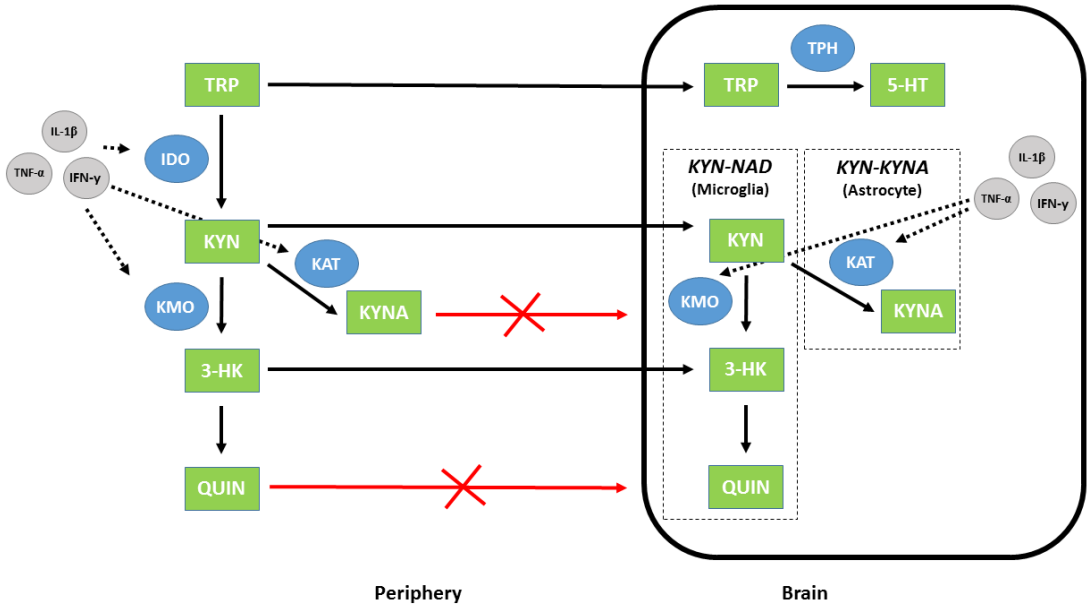

Figure 1: The kynurenine pathway and the influence of proinflammatory mediators

Tryptophan which is not transported across the blood brain barrier (BBB) for the synthesis of serotonin (5-HT) can be degraded in the periphery by indoleamine 2,3-dioxygenase (IDO) into kynurenine (KYN). KYN may then be metabolized within the periphery or after entering the brain along one of two distinct branches to produce either kynurenic acid (KYNA) or 3-hydroxykynurenine (3-HK) and quinolinic acid (QUIN). Pro-inflammatory mediators are capable of up-regulating the activity of enzymes such indoleamine 2,3-dioxygenase (IDO), kynurenine 3-monooxygenase (KMO), and kynurenine aminotransferase (KAT) thereby increasing the production such TRP metabolites. This may result in elevated levels of TRP metabolites with neuroactive properties within the brain.

Materials and Methods

**See Chapter 4 - Overview of Study Design for methodologies regarding participants, randomization, and anti-inflammatory diet*

Measurement of serum inflammatory markers and amino acids:

Blood draws (20ml) were taken from the antecubital vein of each participant at 1pm at each of the 3 testing sessions (baseline, 1 month, and 3 months). Following extraction, the whole blood was allowed to clot for 30 minutes followed by centrifugation at 1000xg for 15 minutes. Serum was extracted and immediately stored at -80°C until

later analysis. Inflammatory mediators of interest included the pro-inflammatory cytokines IL-2, IL-1β, IL-6, TNF-α, IFN-y, the acute phase protein CRP, as well as the anti-inflammatory cytokines IL-4, IL-10, and IL-1RA. Amino acids of interest included tryptophan, kynurenine, phenylalanine, tyrosine, and branched-chain amino acids (valine, leucine, isoleucine). This allowed for the assessment of TRP concentrations as well as the TRP/LNAA ratio (an indicator of TRP availability). Kynurenine levels were also analyzed for the assessment of the KYN/TRP ratio (an indicator of IDO regulation). Analysis of pro and anti-inflammatory cytokines was performed in triplicate via the Magpix Multiplex system (EMD Millipore, MA, U.S.A) and analyzed using Luminex software. CRP was analyzed in triplicate and quantified via enzyme-linked immunosorbent assay (R&D systems, Minneapolis, U.S.A.). TRP, Phe, TYR, BCAA, and KYN were analyzed in triplicate and quanitified via enzyme-linked immunosorbent assay (Immunodiagnostik, Bensheim, Germany; Labor Diagnostika, Nordhorn, Germany).

Assessment of cognitive function:

Participants were asked to perform the California Verbal Learning Test (CVLT) as an indication of verbal learning and memory. This test consisted of 2 word lists each consisting of 4 categories of 4 words, for a total of 16 words per list. The test began with the learning trial whereby the examiner read aloud the first list (List A) of words. These words were announced at one-second intervals, in a fixed order, over 5 learning trials. Following each trial, the participant was asked to recall as many words as possible in any order, including those recalled on previous trials. Following these 5 learning trials, a single trial of an interference list (List B) was presented and participants were asked to immediately recall as many words as possible from this new list. This list (List B)

consisted of 2 shared categories from List A as well as 2 unshared categories. Following the presentation and recall of List B, short delay recall of list A was performed whereby participants were asked once again to recall as many words as possible from List A, both freely (with no help from the examiner), and with semantic cues, whereby participants were told the category (eg. types of vegetables). Following a 20-minute delay, long-delay recall of list A was assessed, once again first freely, and then with semantic cues. Finally, a recognition task was performed whereby participants were verbally presented with 44 words consisting of 16 target words (those from List A) as well as 28 distractor words (those from List B and off-list words). Following the presentation of each word, participants were asked to identify whether or not the word was from List A.

Cognitive performance was examined based on the following: (1) scores from trial 1 of List A, as an indication of short-term memory, (2) the learning slope (slope of scores across the initial 5 trials of List A), as an indication of the rate of learning (3) scores of long-delay free recall, as an indication of longer-term memory, and (4) errors, including the total number of intrusions (incorrect off-list words) and repetitions (both incorrect and correct) across all trials.

Statistical analysis:

Two-way (group x time) repeated measures ANOVA were performed to investigate possible changes in scores of the CVLT across the 3 testing sessions (baseline, 1 month, 3 month). All CVLT subtests were calculated as a T-score (M=50; SD=10). Two-way repeated measures ANOVA were also performed for the proinflammatory cytokine TNF-α and the amino acids TRP, TYR, KYN and the TRP/LNAA ratio. As the remaining inflammatory mediators and amino acids were not

normally distributed, non-parametric analyses were performed. A Friedman's test of differences among repeated measures (baseline, 1 month, 3 month) for the treatment group and control was performed. If the Friedman's test resulted in a significant value, a Wilcoxon signed-rank test was then performed to provide specific information regarding which time points were significantly different from one another. Finally, A Mann-Whitney test was performed on change scores (3-month - baseline) between groups to establish if the change experienced in inflammatory mediators and amino acids significantly differed between groups. These data are expressed as means ± standard deviations. Statistical significance was set at $p \leq 0.05$ for all tests.

Results

All participants from both the treatment and control group completed the entire 3-month duration of the study and were included in the analysis. No adverse events were reported. The participants' overall compliance to the diet was assessed based on the average of the 3 diet records during the study (1 month, 2 months, and 3 months). One participant completed all three testing sessions but failed to produce the 2-month and the 3-month diet record. This participant had a dietary compliance over the first month of 92%. All other participants completed each of the required diet records and overall compliance ranged from 70-100%, with a mean compliance of 89%. A detailed analysis regarding specific diet compliance data will be presented elsewhere.

Change in CVLT Scores:

Changes in CVLT scores are shown in Table 2. No significant group x time interactions were observed for List A, Trial 1 free recall ($p=0.48$, Cohen's $d=0.04$), learning slope coefficient ($p=0.46$, Cohen's $d=0.04$), or long delay free recall ($p=0.83$,

Cohen's d=0.01). When comparing errors, a significant time x interaction was observed for total intrusions (p=0.03, Cohen's d=0.17), and a trend was observed for total repetitions (p=0.07, Cohen's d=0.14). Post hoc analysis for total intrusions revealed a significant improvement for the treatment group from baseline to 3 month (p=0.05), while there was no significant change in the control group at any time point.

Table 2: Change in CVLT Scores

	Treatment (n=12)			Control (n=8)			P-value
	Baseline	**1 Month**	**3 Month**	**Baseline**	**1 Month**	**3 Month**	
Trial 1, List A Free Recall	6.1±1.2	7.2±1.5	8.3±1.7	6.0±1.7	7.0±2.2	7.3±2.1	0.48
Learning Slope Coefficient	1.1±0.5	1.2±0.4	0.9±0.7	1.5±0.8	1.2±0.4	1.0±0.5	0.46
Long Delay Free Recall	9.8±3.1	11.2±3.3	11.8±2.9	10.6±3.3	11.6±2.9	12.1±4.1	0.83
Total Intrusions	5.3±4.7	3.1±4.1	2.9±3.4*	5.0±4.2	6.5±8.0	12.0±14.6	0.03
Total Repetitions	4.9±4.3	3.4±3.7	4.8±3.7	5.1±4.5	8.8±6.3	4.5±3.5	0.07

All results are shown as mean ± SD.
P-values correspond to group x time interactions
**Significantly different from baseline with a P value ≤ 0.05*

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Change in Inflammatory Mediators:

Changes in serum levels of inflammatory mediators are shown in Table 2 of Chapter 5. When considering a proinflammatory composite score (average of IL-2, IL-6, IL-1β, TNF-α, and IFNy), the Mann-Whitney test indicated that the change scores (3-month - baseline) were significantly different between the treatment group and the control group (U=13.0, p=0.01). The Friedman test showed that there was a statistically significant reduction in the proinflammatory composite scores in the treatment group (x2 = 6.50, p=0.04), but no significant change in the control group (x2=5.25, p=0.07). Post hoc analysis performed with the Wilcoxon signed-rank test showed significant reductions in the treatment group from both baseline to 1 month and baseline to 3 months (z=-2.197, p=0.03; z=-2.275, p=0.02 respectively). When analyzing each cytokine separately, the Mann-Whitney test indicated that the change scores (3-month - baseline) were significantly different between the treatment group and the control group for IFN-y (U=13.0, p=0.01.), IL-1β (U=14.0, p=0.01), and IL-2 (U=12.0, p=0.01) and showed a trend for CRP (U=27.0, p=0.10). The Friedman test showed that in the treatment group there was a statistically significant reduction in IFN-y (x2=8.67, p=0.01), IL-1β (x2=17.78, p<0.01), IL-6 (x2=6.17, p<0.05), and a trend for CRP (x2=4.5, p=0.10). The Friedman test showed no statistically significant reductions for any inflammatory mediator in the control group. Post-hoc analysis performed with the Wilcoxon signed-rank test showed significant reductions in the treatment group for IFN-y from baseline to 1 month and baseline to 3 months (z=-2.275, p=0.02; z=-2.510, p=0.01 respectively), as well as significant reductions in the treatment group for IL-1β from baseline to 1 month and baseline to 3 months (z=-3.059, p<0.01; z=-2.934, p<0.01 respectively), and a

significant reduction in the treatment group for IL-6 from baseline to 1 month, and a trend from baseline to 3 months (z=-2.275, p=0.02; z=-1.726, p=0.08 respectively). Two-way repeated measures ANOVA were performed for the normally distributed mediator, TNF-α which showed a trend towards a group x time interaction (p=0.10, Cohen's d=0.12)

Change in Serum Amino Acids:

Changes in serum amino acids are shown in Table 3 of Chapter 5. Two-way repeated measures ANOVA were performed for the normally distributed amino acids. There was a significant group x time interaction for the TRP/LNAA ratio (p=0.04, Cohen's d=0.90). Post hoc analysis showed a trend towards a reduction in TRP/LNAA in the treatment group from baseline to 3 months (p=0.06). Post hoc analysis showed no change in the control group at any time point. KYN showed a trend towards a group x time interaction (p=0.06, Cohen's d=0.81). A Mann-Whitney test was performed on the change scores for non-normally distributed amino acids. Change scores (3-month - baseline) for the KYN/TRP ratio were shown to be significantly different between the treatment and control groups (U=20.0, p=0.03). A Friedman's test was performed to test for significant changes among repeated measures (baseline, 1 month, 3 months) for the treatment group and control group. No significant change in the KYN/TRP ratio was found in the treatment (x2=2.09, p=0.35) or control (x2=4.75, p=0.09) group.

Discussion

The present study was the first to examine the influence of reducing chronically elevated levels of inflammation on cognitive function *in-vivo*, in humans with spinal cord injury. The intervention successfully reduced levels of pro-inflammatory mediators but

only achieved significant improvement in total intrusions. It was hypothesized that a significant reduction in pro-inflammatory mediators would be sufficient to induce alterations in neuroactive compounds of the kynurenine pathway resulting in corresponding improvements in memory. Significant group differences were found for the change in the TRP/LNAA ratio (significant group x time interaction) as well as the KYN/TRP ratio (significant Mann-Whitney test of change scores). The magnitude of change in these ratios, however, did not reach statistical significance in either the treatment or control group. It may be possible that the significant reductions obtained in pro-inflammatory mediators were not considerable enough to induce substantial alterations in IDO regulation and therefore did not allow for meaningful changes in corresponding amino acids. This may explain, in part, the lack of change in scores of memory and verbal learning related to scores of Trial 1, List A, Learning Slope Coefficient, and Long Delay Free Recall.

The lack of such changes may also be attributable to previously developed and potentially irreversible hippocampal atrophy. The TRP metabolite 3-HK is able to cross the BBB from the periphery and induce oxidative damage via the production of reactive oxygen species following an interaction with the enzyme xanthine oxidase.[27] Additionally, 3-HK may be further metabolized once in the brain into the non-BBB transportable metabolite QUIN. QUIN is a potent agonist of NMDA receptors which are densely populated on the hippocampus. Chronically elevated concentrations of QUIN within the brain are therefore capable of inducing excitotoxicity by causing an over-activation of NMDA receptors resulting in an increased influx of calcium ions and corresponding neuronal damage.[28] This may also further contribute to the production of

free radicals and oxidative stress brought on by 3-HK. Together, these mechanisms may contribute to hippocampal atrophy stemming from a chronic elevation in inflammatory mediators. Hippocampal volume loss in the form of reduced grey matter density has been demonstrated in individuals with major depressive disorder (also a chronic inflammatory condition) and has been shown to correlate with reduced scores in verbal recognition memory.[29] As the sample of the current study consisted of individuals with chronic SCI, it may be possible that years of chronic inflammation resulted in hippocampal atrophy which contributed to deficits in memory and verbal learning which could not be attenuated by an acute reduction in inflammation.

The significant improvement in intrusions may be due to the fact that the ability to recognize the source of information (e.g. List A versus List B) and avoid incorrect responses is reflective of frontal lobe function rather than hippocampal function. Accordingly, the frontal lobe has been suggested to play an important role in source memory and the ability to avoid distractor or lure items in cognitive tests.[30] This has been demonstrated in individuals with frontal lobe damage whereby unusually high numbers of false recognitions have been reported on recall tests.[31,32]

In the current study, it may be possible that the frontal lobe was more responsive to the anti-inflammatory intervention compared to the hippocampus. Specifically, the hippocampus has been shown to be susceptible to inflammation-induced excitotoxicity and eventual atrophy, which may make it less responsive to changes in inflammation. The frontal lobe may however be influenced by alternative mechanisms. For example, frontal lobe dysfunction has been demonstrated in individuals with cardiovascular disease (a condition associated with a state chronic inflammation) and has been shown to be

correlated with reduced cerebral blood flow (CBF) and elevated levels of CRP.[33] A reduction in cerebral blood flow may be induced by inflammatory mediators such as CRP by means of their ability to cause a reduction in vasodilators such nitric oxide as well their contribution to plaque formation. Although CBF was not assessed in the current study, previous studies have suggested a relationship whereby diminished CBF and cerebral hypoperfusion may contribute to cognitive deficits in individuals with paraplegia and tetraplegiea.[8] Such speculation may explain the link between the reduction in inflammation and intrusion scores, however, further studies are required to elucidate such a potential effect.

Several potential study limitations should be noted. Although a significant reduction in pro-inflammatory mediators was achieved, it is unclear how large of reduction may be necessary to induce potential changes in cognition. It may be possible that larger reductions could have had a greater influence. Additionally, as hippocampal volume and CBF were not assessed in the current study, it is not possible to claim that such influences were responsible for the lack of effect. In terms of generalizability, our sample was quite representative of the SCI population in Canada, regarding age, level of injury[34] and the levels of inflammation demonstrated were comparable to those previously reported for this population.[9,35,36] It may be necessary however, for future studies to evaluate and control for hippocampal volume when assessing populations that have undergone long periods of chronic inflammation.

Conclusion

The present study demonstrated an improvement in intrusions while other indices of verbal learning and memory remained unchanged despite a significant reduction of

proinflammatory mediators in a population with SCI. These results suggest that reductions in inflammation to this magnitude may not be sufficient to induce meaningful alterations in related neuroactive compounds or corresponding changes in cognition. It may be possible that the ability to avoid incorrect responses is the most sensitive to change, thereby explaining the significant improvement in intrusions. As it is possible that underlying hippocampal atrophy may have contributed to the lack of improvements in most aspects of the test, it will be necessary for future studies to evaluate hippocampal volume when assessing the efficacy of anti-inflammatory interventions for the treatment of cognitive impairment.

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Conflict of Interest:

The authors declare no conflict of interest.

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Chapter 8

Manuscript 3

Targeting Inflammation as a Treatment Modality for Neuropathic Pain in Spinal Cord Injury: A Randomized Clinical Trial

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Abstract

Study Design: This study was a randomized, parallel-group, controlled clinical trial (NCT02099890).

Objectives: The purpose of the present study was to examine the effectiveness of an anti-inflammatory intervention as a treatment for neuropathic pain following spinal cord injury (SCI).

Setting: Participants were recruited from the Niagara region of Ontario Canada

Methods: Twenty participants with varying levels and severities of SCI, were randomized (3:2) to either a 12-week anti-inflammatory diet, or control group. Outcome measures consisted of self-determined indices of pain as assessed using the neuropathic pain questionnaire (NPQ), and markers of inflammation as assessed by various pro and anti-inflammatory cytokines, as well as the eicosanoids PGE2 and LTB4.

Results: A significant group x time interaction was found for sensory pain scores ($p<0.01$). A Mann-Whitney test revealed that the change scores (3 month-baseline) were significantly different between groups for IFNy ($U=13.0$, $p=0.01$.), IL-1β ($U=14.0$, $p=0.01$), and IL-2 ($U=12.0$, $p=0.01$). A Friedman test revealed the treatment group had a significant reduction in IFNy ($x^2=8.67$, $p=0.01$), IL-1β ($x^2=17.78$, $p<0.01$), IL-6 ($x^2=6.17$, $p<0.05$), while the control group showed no significant change in any inflammatory mediator. A step-wise backward elimination multiple regression analysis showed that the change in sensory neuropathic pain was a function of the change in the proinflammatory cytokines IL-2, and IFN-y, as well as the eicosanoids PGE2 ($R=.689$, $R^2=.474$).

Conclusion: Overall the results of the study demonstrate the efficacy of targeting inflammation as a means of treating neuropathic pain in SCI, with a potential mechanism relating to the reduction in proinflammatory cytokines and PGE2.

Trial Registration: ClinicalTrials.gov ID: NCT02099890

Keywords

Neuropathic Pain; Spinal Cord Injury; Inflammation; Anti-inflammatory Diet

Introduction

Damage to the nervous system may result in persistent or permanent changes to nociceptive thresholds resulting in a unique pain state characterized by allodynia and/or hyperalgesia[1]. This form of pain, known as neuropathic pain, effects an estimated 29-75% of the spinal cord injured (SCI) population[2]. Such a condition can be severely debilitating following SCI and may contribute to inactivity and related metabolic conditions, as well as behavioral disorders such depression, anxiety, and insomnia[3]. Neuropathic pain is notoriously difficult to treat and no current treatment has been consistently proven as universally effective, predictable and safe for long-term use[4]. Based on the widespread potential to impact quality of life, it is imperative to gain a better understanding of the underlying mechanisms of neuropathic pain and explore novel treatments.

Traditionally, neuropathic pain has been viewed as a purely neuronal issue, stemming from structural damage to the neuron itself. However, it is now well-established that supporting glial cells and the environment with which the nociceptor interacts are important factors[5]. A number of inflammatory mediators are known to reduce the nociceptive threshold, resulting in symptoms of hyperalgesia[5]. Proinflammatory cytokines such as IL-1β, IFN-y, IL-6, and TNF-α have been proposed to induce algesic effects by both direct[6] and indirect[7] (prostaglandin-dependent) influences. The inflammatory etiology of neuropathic pain is further supported based on evidence that analgesic effects have been produced following the reduction of pro-inflammatory cytokine concentrations. This has been demonstrated in animal models following the

administration of antibodies against TNF-α[8] as well as in transgenic animal models with impaired IL-1β production[9,10].

Pharmacological treatment strategies are currently the most heavily relied upon form of neuropathic pain management. Studies have, however, shown mixed results concerning the efficacy, universality, and associated side effects of treatments such as tricyclic antidepressants, SSRI's, cannabinoids, anticonvulsants, and opioids[4]. This inconsistency across studies may be due, in part, to variability in protocols (e.g. duration, dosage) but may also relate to the varied etiological basis across different participants. A better understanding of the various mechanisms at play and a more advanced mechanism-based classification system will be critical to enhance the specificity and efficacy of current pharmacological treatments. This may contribute to the development of pharmacological treatments which target the underlying mechanisms responsible for neuropathic pain as opposed to focusing solely on symptom relief.

An alternative to traditional pharmaceutical treatments may be the implementation of anti-inflammatory interventions. Such strategies would target the common inflammatory mechanisms which underlie various etiologies thereby having the potential to provide a more universally applicable treatment option. Further, if such strategies prove effective, it may be possible to produce such anti-inflammatory effects via simple lifestyle alterations without the use of pharmaceuticals. This may help to reduce the reliance on traditional pharmaceuticals thereby helping to avoid associated side effects. Appropriate dietary alterations including foods and supplements with established anti-inflammatory benefits have been shown to effectively reduce inflammation via a number of mechanisms ranging from altered gene transcription,

changes in cell membrane composition, improved enzyme regulation, as well as improvements in metabolic heath and body composition[11].

Dietary needs, including nutrient requirements and caloric intake, change drastically following SCI, and are often not adequately met. Diet is therefore, likely a substantial contributing factor to the chronic inflammation typically observed following SCI[12,13]. A dietary intervention consisting of foods and supplements with established anti-inflammatory benefits was therefore utilized in the current study with the intention of reducing inflammation for the purpose of studying the effect on neuropathic pain. It was hypothesized that by reducing chronically elevated levels of inflammatory mediators, corresponding reductions in neuropathic pain would be achieved.

Materials and Methods

**See Chapter 4 - Overview of Study Design for methodologies regarding participants, randomization, and anti-inflammatory diet*

Measurement of serum inflammatory markers:

Blood draws (20ml) were taken from the antecubital vein of each participant at 1pm at each of the 3 testing sessions (baseline, 1 month, and 3 month). Following extraction, the whole blood was allowed to clot for 30 minutes followed by centrifugation at 1000xg for 15 minutes. Serum was extracted and immediately stored at -80°C until later analysis. Inflammatory mediators of interest included the pro-inflammatory cytokines IL-2, IL-1B, IL-6, TNF-alpha, IFN-y, acute phase protein CRP, eicosanoids PGE2 and LTB4, as well as the anti-inflammatory cytokines IL-4, IL-10, and IL-1RA. Both pro and anti-inflammatory cytokines were assessed due to findings that painful neuropathy has been shown to be associated with elevated levels of proinflammatory

mediators and reduced levels of anti-inflammatory mediators while non-painful neuropathy has shown the opposite.[12,15] Improvements in neuropathic pain may therefore relate to reductions in proinflammatory mediators and/or elevations in anti-inflammatory mediators. Analysis of pro and anti-inflammatory cytokines was performed in triplicate via the Magpix Multiplex system and analyzed using Luminex software. CRP, PGE2, and LTB4 were analyzed in triplicate and quantified via enzyme-linked immunosorbent assay (R&D systems, Minneapolis, U.S.A.).

Assessment of neuropathic pain:

Participants were asked to complete the Neuropathic Pain Questionnaire (NPQ) at each of the 3 testing sessions, as a means of assessing self-reported neuropathic pain. The questionnaire consisted of 32 items pertaining to 3 unique categories including sensory items, affective items, and sensitivity items. Sensory items were those related to the specific type and severity of pain felt (e.g. degree of burning, stabbing, throbbing, etc.), affective items referred to those related to how the pain affected the participant in daily life (e.g. how irritating is your usual pain?) and sensitivity items related to how various stimuli may act to increase pain (e.g. increased pain due to heat). Participants were asked to rate their pain numerically on a scale from 0-100 whereby 0 indicated the complete absence of pain and 100 indicated the worst pain imaginable. Scores from each of the 3 categories were averaged for use in statistical analysis.

Statistical analysis:

Two-way (group x time) repeated measures ANOVA were performed to investigate possible changes in pain scores related to sensory and affective pain across 3 testing sessions (baseline, 1 month, 3 month). Two-way repeated measures ANOVA were

also performed for the proinflammatory cytokine TNF-α and the eicosanoid PGE2. As the remaining inflammatory mediators as well as sensitivity pain scores were not normally distributed, non-parametric analyses were performed. A Friedman's test of differences among repeated measures (baseline, 1 month, and 3 month) for the treatment group and control was performed. If the Friedman's test resulted in a significant value, a Wilcoxon signed-rank test was then performed to provide specific information regarding which time points were significantly different from one another. Finally, A Mann-Whitney test was performed on change scores (3-month - baseline) between groups to establish if the change experienced significantly differed between groups. These data are expressed as means ± standard deviations. Correlations between changes in inflammatory mediators, and neuropathic pain scores were assessed by means of Pearson's r correlation. Statistical significance was set at $p \leq 0.05$ for all tests. The proportional contribution of the change in each inflammatory mediator to the change in pain score was evaluated using a step-wise backwards elimination multiple regression. Levels of F to enter and F to remove were set to correspond to p-levels of .05 and .10 respectively.

Results

All participants from both the treatment and control group completed the entire 3-month duration of the study and were included in the analysis. No adverse events were reported. The participants' overall compliance to the diet was assessed based on the average of the 3 diet records during the study (1 month, 2 months, and 3 months). One participant completed all three testing sessions but failed to produce the 2-month and the 3-month diet record. This participant had a dietary compliance over the first month of 92%. All other participants completed each of the required diet records and overall

compliance ranged from 70-100%, with a mean compliance of 89%. A detailed analysis regarding specific diet adherence data will be presented elsewhere.

Change in pain scores:

Changes in sensory, affective, and sensitivity pain scores are shown in table 2. There was a significant group x time interaction for the sensory component of the self-reported neuropathic pain scores ($p<0.01$; cohen's $d=1.29$). Post hoc analysis showed a significant reduction in sensory scores in the treatment group from both baseline to 1 month, as well as baseline to 3 months ($p=<0.01$ and $p=.01$ respectively). Post hoc analysis showed a significant increase in sensory scores in the control group from baseline to 1 month ($p=.04$) but no significant change from baseline to 3 months ($p=.210$) No significant group x interaction was found for the affective component of the self-reported neuropathic pain scores ($p=0.17$; cohen's $d=0.63$). In regard to the non-parametric analysis of sensitivity scores, the Mann-Whitney test indicated that change scores of sensitivity pain were not significantly different between the treatment group and control group ($U=36.0$, $p=0.35$). The Friedman test showed that there was no significant change in sensitivity pain scores for either the treatment group ($x2=3.38$, $p=0.19$) or control group ($x2=0.09$, $p=0.96$) across testing sessions.

Table 2: Change in NPQ Scores

	Treatment (n=12)			Control (n=8)			2-Way ANOVA (p-value)	Mann-Whitney (p-value)	Friedman [Treat.] (p-value)
	Baseline	**1 Month**	**3 Month**	**Baseline**	**1 Month**	**3 Month**			
Sensory Score	32.8±23.4	23.4±20.2**	19.8±15.8**	18.1±17.2	25.2±22.2*	21.3±20.1	<0.01	--	--
Affective Score	34.7±28.6	24.3±21.9	21.2±19.0	27.5±24.4	20.1±23.0	23.7±25.3	0.18	--	--
Sensitivity Score	26.8±26.1	22.6±21.2	22.6±20.7	29.7±32.9	33.7±26.9	32.6±26.3	--	0.35	0.19

All results are shown as mean ± SD. P-values correspond to group x time interactions, Mann-Whitney change scores, and Friedman scores for treatment group respectively (Friedman scores for control group not shown).
**Significantly different from baseline with P value <0.05*
***Significantly different from baseline with P value <.01*

Change in Inflammatory Mediators:

Changes in serum levels of inflammatory mediators are shown in Table 2 of Chapter 5. When considering a proinflammatory composite score (average of IL-2, IL-6, IL-1β, TNF-α, and IFNy), the Mann-Whitney test indicated that the change scores (3-month - baseline) were significantly different between the treatment group and the control group (U=13.0, p=0.01). The Friedman test showed that there was a statistically significant reduction in the proinflammatory composite scores in the treatment group (x2 = 6.50, p=0.04), but no significant change in the control group (x2=5.25, p=0.07). Post hoc analysis performed with the Wilcoxon signed-rank test showed significant reductions in the treatment group from both baseline to 1 month and baseline to 3 months (z=-2.197, p=0.03; z=-2.275, p=0.02 respectively). When analyzing each cytokine separately, the Mann-Whitney test indicated that the change scores (3-month - baseline) were significantly different between the treatment group and the control group for IFN-y (U=13.0, p=0.01.), IL-1β (U=14.0, p=0.01), and IL-2 (U=12.0, p=0.01) and showed a trend for CRP (U=27.0, p=0.10). The Friedman test showed that in the treatment group there was a statistically significant reduction in IFN-y (x2=8.67, p=0.01), IL-1β (x2=17.78, p<0.01), IL-6 (x2=6.17, p<0.05), and a trend for CRP (x2=4.5, p=0.10). The Friedman test showed no statistically significant reductions for any inflammatory mediator in the control group. Post-hoc analysis performed with the Wilcoxon signed-rank test showed significant reductions in the treatment group for IFN-y from baseline to 1 month and baseline to 3 months (z=-2.275, p=0.02; z=-2.510, p=0.01 respectively), as well as significant reductions in the treatment group for IL-1β from baseline to 1 month and baseline to 3 months (z=-3.059, p<0.01; z=-2.934, p<0.01 respectively), and a

significant reduction in the treatment group for IL-6 from baseline to 1 month, and a trend from baseline to 3 months (z=-2.275, p=0.02; z=-1.726, p=0.08 respectively). Two-way repeated measures ANOVA were performed for the normally distributed mediators TNF-α and PGE2 and showed trends towards group x time interactions (p=0.10; p=0.07 respectively).

Relationship between inflammatory mediators and indices of pain:

Results from the multiple regression are shown in table 4. To help elucidate a potential mechanism between the reduction in neuropathic pain scores and inflammatory mediators a step-wise backwards multiple regression analysis was performed. When assessing the change in sensory pain score as the outcome variable, results from the regression analysis provided partial confirmation of the research hypothesis that change in sensory neuropathic pain is a function of the change in proinflammatory cytokines and eicosanoids. The 3 variable model included the proinflammatory cytokines IL-2 and IFN-y, as well as the eicosanoid PGE2 (R=.689, R2=.474). The overall F statistic for the model was 4.811, df=3,16, p=.014. Standardized beta weights were -.730 for IL-2, .544 for IFN-y, and .526 for PGE2. When assessing the change in affective pain score as the outcome variable only PGE2 remained in the model (R=.558, R2=.312). The overall F statistic for the 1 variable model was 8.145, df=1.18, p=.011, and the standardized beta weight was .558. Lastly, when assessing the change in sensitivity score as the outcome variable, a 3 variable model including the proinflammatory cytokines IL-1B and IL-2 as well as the eicosanoid PGE2 remained (R=.715, R2=.511). The overall F statistic for the 3 variable model was 5.580, df=3,16, p=.008. Standardized beta weights were .491 for IL-1B, -.666 for IL-2 and .378 for PGE2.

Table 4: Multiple Regression

Outcome Variable	Predictor Variables	b	SE-b	Beta	t	p value	Tolerance	VIF
Sensory	IL-2	-1.386	.483	-0.73	-2.87	0.01	0.51	1.97
(R=.69, R^2=.47)	IFN-y	.179	.081	0.54	2.21	0.04	0.52	1.91
	PGE2	.022	.008	0.53	2.79	0.01	0.92	1.08
Affective	PGE2	.02	.01	0.56	2.85	0.01		
(R=.56, R^2=.31)								
Sensitivity	IL-1B	9.67	4.93	0.49	1.96	0.07	0.49	2.05
(R=.72, R^2=.51)	IL-2	-1.49	.49	-0.67	-3.07	0.01	0.65	1.54
	PGE2	.02	.01	0.38	1.8	0.09	0.69	1.45

Backward stepwise multiple regression analysis of the relationship between the change in neuropathic pain scores and the change in inflammatory mediators

Discussion

The present study successfully obtained reductions in inflammatory mediators and scores of sensory neuropathic pain in individuals with chronic SCI by means of dietary alterations. The relationship between changes in inflammatory mediators and changes in sensory neuropathic pain scores was assessed via a step-wise, backwards elimination multiple regression analysis. It was demonstrated that approximately 47% of the change in sensory neuropathic pain scores could be explained by the change in three inflammatory mediators including IL-2, IFN-y, and PGE2. Both IFN-y and PGE2 demonstrated positive relationships whereby a one-unit decrease in these mediators, was related to a respective .544 and .526-unit decrease in sensory neuropathic pain scores. IL-2 demonstrated a negative relationship whereby a one-unit increase was related to a .730-unit decrease in sensory neuropathic pain scores. Such findings may be explained by the

various peripheral and central mechanisms through which these mediators have been shown influence pain.

Under non-pathological conditions, free nerve endings in the periphery detect painful mechanical, thermal, or chemical stimuli and generate nerve impulses which then travel along afferent A-delta, or C fibers to the dorsal horn of the spinal cord. At this point A-delta fibers synapse with second order neurons (while C fibers first synapse with interneurons) before ascending along the spinothalamic tract to the thalamus and somatosensory cortex whereby the magnitude and location of pain is processed. Under pathological conditions, chronic inflammation may influence pain signaling at different points along this pathway.

PGE2 has been shown to be a key factor in inflammatory-evoked pain by means of inducing sensitization of peripheral nociceptors as well as by inducing central changes concerning the processing of spinal nociceptive input[16]. Peripherally, PGE2 acts on corresponding receptors (EP) on the nociceptor which causes a protein kinase A (PKA)-mediated phosphorylation of sodium channels thereby causing peripheral sensitization[16]. PGE2 has also been shown to induce similar effects centrally by inducing membrane depolarization of dorsal horn neurons leading to action potential generation[17]. There is also evidence that PGE2 may target the inhibitory glycine receptor thereby reducing inhibitory glycinergic synaptic transmission. This provides a second mechanism by which PGE2 may facilitate transmission of nociceptive input through the dorsal horn of the spinal cord[18].

IFN-y has also been shown to induce central effects which contribute to the enhancement of neuropathic pain. Spinal microglia express receptors for IFN-y. Once

activated by IFN-y they have been shown to induce the production of bioactive factors such as cytokines and neurotrophic factors[19,20] thereby influencing the excitability of the dorsal horn pathway[21] and injury-induced pain behaviours[22]. Through this process IFN-y is able to indirectly enhance pain processing in the dorsal horn and influence neuropathic pain.

The negative relationship found between IL-2 and sensory pain scores can be explained by previous reports which have demonstrated anti-nociceptive properties of IL-2. Pain transmission can be depressed by means of inducing hyperpolarization, thereby reducing neuronal excitability, and depressing the release of nociceptive neurotransmitters[23]. Free intracellular calcium (Ca2+) plays a key role in the release of nociceptive neurotransmitters such as substance P from the presynaptic neuron[24,25]. It is through this mechanism that opioids have been demonstrated to produce their analgesic effects. The activation of opioid receptors has been shown to suppress high threshold Ca2+ currents in rat dorsal root ganglion thereby inducing presynaptic inhibition[26,27]. IL-2 administration has been demonstrated to induce a similar influence on high threshold Ca2+ currents and inhibit the depolarization-evoked increase in intracellular Ca2+ concentration[28]. The fact that administration of the u-opioid antagonist naloxone produces dramatic reductions in this effect suggests that IL-2 may also be producing these effects by acting on opioid receptors[29].

Although speculative, the mechanisms discussed above may explain the link between the dietary-induced reduction in inflammation and the reductions in neuropathic pain scores observed in the current study. The fact that dietary alterations may target the underlying mechanisms of neuropathic pain may explain its effectiveness as a treatment

modality. This could provide some advantage over traditional pharmacological treatments which focus on symptom relief aimed at downstream targets such as those involving the direct reduction of neuronal hyperexcitability. Such strategies have demonstrated mixed results in terms of efficacy, universality, and associated side effects. Examples include the use of tricyclic antidepressants which have shown efficacy for central pain[30], but also a lack of effect for HIV neuropathy[31,32], or chemotherapy-induced neuropathic symptoms[33,34]. SSRI's have also been shown to produce only weak analgesic effects[35–37]. The use of cannabinoids has been shown to relieve peripheral neuropathic pain in some studies[38–40], but has shown no effect in painful poly-neuropathy[41]. Anticonvulsants such as Gabapentin have been shown to be effective for the relief of painful polyneuropathy[42], but have also demonstrated a lack of effect in several studies[30,43–45]. Finally, opioids have been demonstrated, fairly consistently to benefit symptoms of neuropathic pain[46–50], however, the risk of addiction and gastrointestinal side effects may make long-term use inappropriate[51]. The fact that the etiological basis of neuropathic pain may be highly variable from one individual to another makes it difficult to establish a universally effective treatment and may explain the marginal effectiveness of many drug trials. While a particular drug treatment may demonstrate a large effect on a small subgroup of participants it also commonly shows a complete lack of effect in others, resulting in minimal overall efficacy. As dietary alterations are capable of targeting the underlying inflammatory mechanisms of neuropathic pain they may provide a more universal treatment option.

In addition to the potential for a greater universality and reduction in undesirable side-effects, anti-inflammatory strategies such as that utilized in the current study, may

provide benefits comparable in magnitude to traditional pharmacological methods. Gabapentin is currently among the most promising pharmaceutical treatments for neuropathic pain. Studies which examined an 8-week administration of gabapentin have demonstrated reductions in neuropathic pain scores of 40.6% in individuals with painful diabetic neuropathy[52], 33.3% in individuals with postherpetic neuralgia[53], and 21.1% in individuals with mixed neuropathic pain syndromes[54] (based on an 11-point Likert scale). The 39.6% reduction in sensory neuropathic pain scores achieved in the current study is therefore of comparable or better magnitude to that achieved following gabapentin administration. Further, according to Farrar et al. 2001, a 30% reduction in a pain intensity numerical rating scale (PI-NRS) represents a clinically important difference in pain[55].

Several potential study limitations should be noted. First, the study was only single blinded. While the examiner was blinded to group allocation during all blood analysis, participants were aware of their group assignment. Although placebo supplements could have been provided to the control group, it was not possible to adequately blind participants to all aspects of the diet. The treatment group underwent a highly restrictive diet devoid of any refined, processed, or fried foods while the control group was free to consume such foods, making distinction between groups quite obvious. Second, it is not possible to elucidate the specific mechanisms related to the reductions in inflammation, nor is it possible to discern which aspects of the dietary intervention may have had the strongest effects. It will be necessary for future studies to examine aspects such as transcription factor activity and membrane composition in order to truly elucidate the means by which such interventions act to reduce inflammation and improve

symptoms of neuropathic pain. Third, although our sample was quite representative of the SCI population in Canada, in terms of age, level and severity of injury[56], the results are not necessarily generalizable to other chronic inflammatory populations or all forms of neuropathic pain. It also worth noting that the use of interventions which target inflammation by such means as diet or exercise requires commitment to major lifestyle alterations and it may be possible that improvements are less drastic and occur at a slower rate than that of certain pharmaceuticals. However, given the potential for such anti-inflammatory interventions to provide a more universal aid for neuropathic pain symptoms, (in addition to contributing to a multitude of other health benefits), while lacking the side effects associated with traditional pain medications, there is seemingly little reason not to promote such lifestyle alterations as a treatment option.

Conclusion

The present study demonstrated that it was possible to improve symptoms of neuropathic pain in spinal cord injury by means by reducing levels inflammation. Secondarily, appropriate dietary alterations may be one such intervention strategy which could be used to reduce inflammation and induce such benefits. This influence is worthy of further examination as it may help to reduce the reliance on traditional pain medications and provide a safe, sustainable, and more universally applicable treatment modality.

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The authors declare that no competing financial interests exist.

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Chapter 9

Manuscript 4

Targeting inflammation to influence peripheral somatic nerve function following spinal cord injury: A Randomized Clinical Trial

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Abstract

Study Design: This study was a randomized, parallel-group, controlled clinical trial (NCT02099890).

Objectives: The purpose of the present study was to examine the efficacy of targeting inflammation as a means of improving somatic nerve conduction following spinal cord injury (SCI).

Setting: Participants were recruited from the Niagara region of Ontario Canada

Methods: The study was a randomized, parallel-group, controlled, clinical trial (NCT02099890) whereby 20 participants with varying levels and severities of SCI were randomized (3:2) to either the treatment group, consisting of a 12-week anti-inflammatory diet, or control group. Outcome measures were assessed at baseline, 1-month, and 3-months and consisted of measures of both motor and sensory nerve conduction velocity (NCV) and amplitudes, as well as markers of inflammation as assessed by various pro and anti-inflammatory cytokines.

Results: Despite a significant reduction in inflammation in the treatment group, two-way repeated measures ANOVA showed no significant group x time interaction for motor NCV ($p=0.77$) or M-wave amplitude ($p=0.61$). There was also no significant group x time interaction for sensory NCV ($p=0.32$) or sensory nerve action potential (SNAP) amplitude ($p=0.91$). Further, there were no significant correlations between the change in either motor NCV or M-wave amplitude and the change in any inflammatory mediator of interest.

Conclusion: These results suggest that at physiologically relevant concentrations, inflammatory mediators may not have a substantial influence on peripheral nerve function following SCI. Anti-inflammatory strategies may therefore not provide a suitable treatment option, however, future larger scale are still needed to examine the potential for central effects.

Trial Registration: ClinicalTrials.gov ID: NCT02099890

Keywords

Motor; Sensory; Nerve conduction velocity; Chronic inflammation; Spinal cord injury; Anti-inflammatory

Introduction

A state of chronic inflammation is commonly reported following spinal cord injury (SCI)[1–5]. This persistent inflammatory state, characterized by chronically elevated levels of proinflammatory cytokines, can be attributed to a number of factors which commonly arise following SCI. This population is at a heightened risk for a number acute secondary health complications such as pressure ulcers, as well as urinary tract, and respiratory infections. In addition, the loss of motor function often leads to a more sedentary lifestyle contributing to the risk for the development of a variety of inflammation-inducing metabolic disorders such as obesity, cardiovascular disease and type 2 diabetes. Damage to the spinal cord may also directly result in immune and endocrine dysfunction due to the respective loss of sympathetic innervation of lymphoid organs and dysregulation of the hypothalamic-pituitary-adrenal axis[6].

In addition to concerns related to immune dysregulation and the heighted risk of infection, a primary issue of SCI is the loss of varying degrees of motor and sensory function. Such losses arise from structural damage to neural tracts within the spinal cord whereby an injury affecting all tracts, would result in a motor and sensory complete injury. Interestingly, previous research has demonstrated that it is possible for an injury to present clinically as motor or sensory complete despite the preservation of structurally healthy neural tracts (as shown by MRI). Such injuries are classified as discomplete, and may indicate that some form of non-structurally based influence is inducing deficits in these otherwise healthy tracts [7,8]. Evidence exists to suggest that inflammatory mediators may possess the ability to influence ion channel kinetics and induce channelopathy, resulting in conduction deficits.

A study by Davies et al. (2006) demonstrated in ex-vivo conditions that highly elevated concentrations of different cytokines (e.g. TNF-α) were capable of inducing conduction deficits in a reversible, dose-dependent manner[9]. This relationship has also been demonstrated in-vivo, in humans, under conditions of acute, exercise-induced elevations of inflammatory mediators[10]. Further, a case study performed by Mcdonald et al. (2002) provided some indirect, yet intriguing, results concerning the neural influence of chronically elevated levels of inflammation in humans[11]. In this study, an individual with complete tetraplegia began an intensive diet and exercise program following a 5-year post SCI period in which no observable improvements were achieved. The intervention resulted in a dramatic reduction in clinical events which were matched by significant improvements in scores of both motor and sensory function. Unfortunately, inflammatory mediators were not quantified in this study, however, as a large reduction in events would be expected to coincide with a reduction in inflammation, it is possible to speculate that similar channelopathic mechanisms to that discussed previously[9] may have been responsible.

Despite previous research, suggesting the potential for an inflammatory-induced channelopathy, no study has demonstrated a causal relationship in humans, under physiologically relevant levels of chronic inflammation. If such a relationship exists, it may be possible to induce improvements in motor and/or sensory function in those with SCI via the implementation of anti-inflammatory strategies. The purpose of this study was therefore, to examine the efficacy of targeting inflammation as a means of improving somatic nerve conduction following SCI. Based on the relationship observed between acute elevations in inflammatory mediators and nerve conduction in previous studies, it

was hypothesized that reducing levels of chronic inflammation would result in corresponding improvements in both motor and sensory nerve function.

Materials and Methods

**See Chapter 4 - Overview of Study Design for methodologies regarding participants, randomization, and anti-inflammatory diet*

Measurement of serum inflammatory markers:

Blood draws (20ml) were taken from the antecubital vein of each participant at 1pm at each of the 3 testing sessions (baseline, 1 month, and 3 months). Following extraction, the whole blood was allowed to clot for 30 minutes followed by centrifugation at 1000xg for 15 minutes. Serum was extracted and immediately stored at -80°C until later analysis. Inflammatory mediators of interest included the pro-inflammatory cytokines IL-2, IL-1β, IL-6, TNF-α, IFN-y, the acute phase protein CRP, the anti-inflammatory cytokines IL-4, IL-10, and IL-1RA, and the pro-inflammatory eicosanoid PGE2. Analysis of pro and anti-inflammatory cytokines was performed in triplicate via the Magpix Multiplex system (EMD Millipore, MA, U.S.A) and analyzed using Luminex software. CRP and PGE2 was analyzed in triplicate and quantified via enzyme-linked immunosorbent assay (R&D systems, Minneapolis, U.S.A.).

Assessment of Nerve Conduction:

Motor nerve conduction velocity and M-wave amplitude was assessed using compound motor action potential (CMAP's) recordings by means of stimulation of the median nerve and recording the corresponding motor response of the thenar muscles. Subjects lay in a supine position with the elbow in full extension. Prior to performing the test, the electrode locations were prepared by shaving the skin, removing any dead skin

cells with an abrasive gel, and disinfecting the areas with rubbing alcohol. Surface electrodes were placed in a monopolar configuration with the recording electrode directly over the motor point of the thenar muscles, the reference electrode over the tendon of the interphalangeal joint, and the ground on the palm of the hand. Stimulation of the median nerve was performed distally at the wrist between the flexor tendons of the hand, as well as proximally on the medial side of the biceps brachii. Stimulation intensity was determined by gradually increasing the amplitude until a maximal M-wave was achieved. A stimulation amplitude of 120% of this value was then used during testing. The evoked responses were amplified using a bandwidth of 10Hz-1kHz. A total of 10 trials were taken and averaged for each stimulation site and M-wave onset was used to determine latency. The amplitude of the M-wave was also assessed as an indication of the strength of the motor response. Sweep speed was 2ms per division, sensitivity was 2uV per division, and stimulus duration was 0.2ms at a stimulus rate of 3Hz. Recordings were performed on the Dantec Keypoint EMG (Dantec Medical A/S, Skovlunde, Denmark). Skin temperature was assessed with a surface probe placed over the thenar muscles to ensure consistent temperatures between testing session (MLT422/D Skin temperature probe, AD Instruments, Colorado Springs, USA).

The assessment of sensory NCV and sensory nerve action potential (SNAP) amplitude was performed using an orthodromic electrode configuration. Stimulation was applied using ring electrodes placed just proximal to the second and third metacarpophalangeal joints of the fourth finger. The ground electrode was place on the dorsal surface of the hand. In order to determine an appropriate stimulation amplitude, stimulation was gradually increased until a maximum SNAP amplitude was achieved.

The stimulation utilized during testing was 120% of this maximum SNAP amplitude. A train of 10 impulses was then applied and averaged for the determination of sensory NCV and SNAP amplitude. Sweep was set at 1ms per division, sensitivity was set at 20uV for division, and stimulus duration was set at 0.2ms at a rate of 2Hz. Recordings were once again performed using the Dantec Keypoint EMG.

Statistical analysis:

Two-way (group x time) repeated measures ANOVA were performed to investigate possible changes in scores of motor and sensory NCV and amplitude across 3 testing sessions (baseline, 1 month, 3 month). Two-way repeated measures ANOVA were also performed for the proinflammatory cytokine TNF-α and the eicosanoid PGE2. As the remaining inflammatory mediators were not normally distributed, non-parametric analyses were performed. A Friedman's test of differences among repeated measures (baseline, 1 month, 3 month) for the treatment group and control was performed. If the Friedman's test resulted in a significant value, a Wilcoxon signed-rank test was then performed to provide specific information regarding which time points were significantly different from one another. Finally, A Mann-Whitney test was performed on change scores (3-month - baseline) between groups to establish if the change experienced in inflammatory mediators significantly differed between groups. These data are expressed as means ± standard deviations. Correlations between changes in inflammatory mediators, and measures of nerve conduction were assessed by means of Pearson's r correlation. Statistical significance was set at $p \leq 0.05$ for all tests.

Results

All participants from both the treatment and control group completed the entire 3-month duration of the study and were included in the analysis. No adverse events were reported. The participants' overall compliance to the diet was assessed based on the average of the 3 diet records during the study (1 month, 2 months, and 3 months). One participant completed all three testing sessions but failed to produce the 2-month and the 3-month diet record. This participant had a dietary compliance over the first month of 92%. All other participants completed each of the required diet records and overall compliance ranged from 70-100%, with a mean compliance of 89%. A detailed analysis regarding specific diet adherence data will be presented elsewhere.

Change in Motor and Sensory Nerve Conduction:

Changes in motor NCV and M-wave amplitude are shown in Table 2. No significant group x time interactions were observed for motor NCV ($p=0.77$, Cohen's $d=0.26$) or M-wave amplitude ($p=0.61$, Cohen's $d=0.35$). Change in sensory NCV and SNAP amplitude are shown in Table 2. No significant group x time interactions were observed for sensory NCV ($p=0.32$, Cohen's $d=0.54$) or sensory amplitude ($p=0.91$, Cohen's $d=0.16$).

Table 2: Change in Motor and Sensory NCV and Amplitude

	Treatment			Control		
	Baseline	**1 Month**	**3 Month**	**Baseline**	**1 Month**	**3 Month**
Motor NCV (m/s)	49.8±6.9	49.1±4.0	51.2±5.4	52.0±5.6	52.3±3.5	52.8±6.1
M-Wave Amp. (mV)	4.0±1.5	4.0±2.2	3.3±1.8	5.9±1.6	5.8±2.2	5.9±2.3
Sensory NCV (m/s)	43.5±9.1	42.7±7.7	40.5±6.5	44.1±7.9	45.8±7.4	44.9±8.6
Sensory Amp (μV)	8.5±3.0	6.9±2.0	5.3±1.4	10.9±3.7	9.7±5.1	7.7±3.5

All results are shown as mean ± SD.

Change in Inflammatory Mediators:

Changes in serum levels of inflammatory mediators are shown in Table 2 of Chapter 5. When considering a proinflammatory composite score (average of IL-2, IL-6, IL-1β, TNF-α, and IFNy), the Mann-Whitney test indicated that the change scores (3 month - baseline) were significantly different between the treatment group and the control group (U=13.0, p=0.01). The Friedman test showed that there was a statistically significant reduction in the proinflammatory composite scores in the treatment group (x2 = 6.50, p=0.04), but no significant change in the control group (x2=5.25, p=0.07). Post hoc analysis performed with the Wilcoxon signed-rank test showed significant reductions in the treatment group from both baseline to 1 month and baseline to 3 months (z=-2.197, p=0.03; z=-2.275, p=0.02 respectively). When analyzing each cytokine separately, the Mann-Whitney test indicated that the change scores (3-month - baseline) were significantly different between the treatment group and the control group for IFN-y

(U=13.0, p=0.01.), IL-1β (U=14.0, p=0.01), and IL-2 (U=12.0, p=0.01) and showed a trend for CRP (U=27.0, p=0.10). The Friedman test showed that in the treatment group there was a statistically significant reduction in IFN-y (x2=8.67, p=0.01), IL-1β (x2=17.78, p<0.01), IL-6 (x2=6.17, p<0.05), and a trend for CRP (x2=4.5, p=0.10). The Friedman test showed no statistically significant reductions for any inflammatory mediator in the control group. Post-hoc analysis performed with the Wilcoxon signed-rank test showed significant reductions in the treatment group for IFN-y from baseline to 1 month and baseline to 3 months (z=-2.275, p=0.02; z=-2.510, p=0.01 respectively), as well as significant reductions in the treatment group for IL-1β from baseline to 1 month and baseline to 3 months (z=-3.059, p<0.01; z=-2.934, p<0.01 respectively), and a significant reduction in the treatment group for IL-6 from baseline to 1 month, and a trend from baseline to 3 months (z=-2.275, p=0.02; z=-1.726, p=0.08 respectively). Two-way repeated measures ANOVA were performed for the normally distributed mediator's TNF-α and PGE2 and showed trends towards group x time interactions (p=0.10; p=0.07 respectively).

Correlational Analysis:

Pearson's r correlation coefficients for the change in inflammatory mediators and the change in nerve conduction data are shown in Table 4. Scores related to the change in both motor NCV and motor amplitude were not significantly correlated with the change in any inflammatory mediators. The change in sensory NCV was significantly correlated with only the change in TNF-α (r=.583, p=0.01). The change in sensory amplitude was correlated with the change in IL-1B (r=.576, p=0.01), the change in IL-2 (r=.700, p<.01), the change in CRP (r=.506, p=.03), and the change in PGE2 (r=.568, p=0.01).

Table 4: Correlation Matrix

All Participants				
	Δ*CMAP-NCV*	**Δ*CMAP-AMP***	**Δ*SNAP-NCV***	**Δ*SNAP-AMP***
Δ*Proinflammatory Composite*	-.*386*	-.*205*	.*404*	.*364*
Δ*CRP*	-.116	-.366	.269	.506*
Δ*IL-2*	-.225	-.416	.147	.700**
Δ*IL-6*	-.342	-.381	.444	.297
Δ*IL-1B*	-.133	-.043	.126	.576*
Δ*TNF-α*	-.191	-.034	.583*	-.005
Δ*IFN-y*	-.415	-.401	.384	.334
Δ*Anti-inflammatory Composite*	-.443	-.200	.144	.212
Δ*IL-4*	-.267	-.093	-.269	.323
Δ*IL-10*	-.028	-.070	.070	.039
Δ*IL-1RA*	-.408	-.152	.378	.039
Δ*PGE2*	.194	.305	-.019	.568*

**p≤.05; **p≤ 0.01*
CMAP-NCV: Compound motor action potential nerve conduction velocity; CMAP-AMP: Compound motor action potential amplitude; SNAP: Sensory nerve action potential nerve conduction velocity; SNAP-AMP: Sensory nerve action potential amplitude
Proinflammatory composite consists of a composite score averaging IL-2, IL-6, IL-1B, TNF-α, and IFN-y
Anti-inflammatory composite consists of a composite score averaging IL-4, IL-10, and IL1RA

Discussion:

The present study was the first to examine the influence of reducing chronically elevated levels of inflammation on somatic nerve function in-vivo, in humans. The intervention was successful at obtaining reductions in inflammatory mediators, yet failed to induce any changes in peripheral motor or sensory nerve conduction. As previous research has provided evidence that certain inflammatory mediators may induce

channelopathy under acutely elevated concentrations[9,10], it was hypothesized that similar or even more severe effects would be observed under chronically elevated conditions. Further, if such channelopathic effects were to occur in humans, in-vivo, individuals with SCI may be expected to be particularly at risk due to the high prevalence of both chronic inflammation and somatic nerve deficits.

Inflammatory mediators have been proposed to contribute to such somatic nerve deficits by interfering with neuronal membrane channels and blocking the exchange of ions across the membrane. If sodium (Na+) and potassium (K+) exchange is sufficiently blocked, normal membrane depolarization and/or repolarization may be disrupted thereby causing a reduction in nerve excitability related to motor nerve channelopathy[9,13]. Such an influence would be fitting of the dose-dependent, reversible conduction deficits observed in previous ex-vivo studies[9], but did not seem apparent in the current study.

Inflammatory mediators also have a well-established enhancing effect on nociceptive sensory fibers. While a potential influence on A-β fibers remains unclear, inflammatory mediators are known to sensitize fibers responsible for nociception, such as A-δ, and C fibers, at various points along the nociceptive pathway. Peripherally, inflammatory mediators such as PGE2 may act on corresponding receptors on the nociceptor and induce a protein-kinase A-mediated phosphorylation of sodium channels, thereby causing peripheral sensitization[14]. IL-1β and TNF-α have also been shown to increase excitability in nociceptive neurons and enhance sodium currents [15–17]. As stimulations performed at low frequencies (such as those of the present study) have been shown to result in the recruitment A-β, a-δ, and C fibers [18,19], the SNAP amplitudes and NCV's recorded in the present study would be expected to represent each of these fibers.

The enhancing effect of inflammatory mediators on A-delta, and C fibers may therefore explain the positive correlation between indices of sensory nerve conduction and several inflammatory mediators found in the present study.

Despite these few positive correlations, no significant change in either motor or sensory nerve conduction was achieved, suggesting that under physiologically relevant concentrations, reducing chronically elevated inflammatory mediators may not be sufficient to induce meaningful changes in somatic nerve conduction. No significant group x time interaction was observed for either motor or sensory NCV or amplitude. Further no significant correlations were observed between the changes in either index of motor conduction and the change in any inflammatory mediator. The positive correlations observed between of the change in sensory nerve conduction and the change in several inflammatory mediators are in agreement with previous reports which have demonstrated the relationship between inflammation and nociceptor hyper-excitability[20]. This does not however imply any channelopathic effect of inflammatory mediators on non-nociceptive sensory fibers. These results are clinically relevant as it may suggest that interventions which target inflammation may not be an effective strategy in terms of improving motor and/or sensory function in individuals with SCI.

Study Limitations

Several potential study limitations should be noted. Although no changes in motor or sensory nerve conduction were observed in the present study, some degree of influence may still be possible under particularly severe levels of inflammation. It may also be possible that other inflammatory mediators, not assessed in the current study may have demonstrated a stronger relationship with measures of somatic nerve conduction.

However, in terms of generalizability, our sample was quite representative of the SCI population in Canada, regarding age, level, and severity of injury[21] and the levels of inflammation demonstrated were comparable to those previously reported for this population[1,3,22]. In addition, the current study examined a wide array of both pro and anti-inflammatory cytokines. As such, we feel confident that these results are representative of the average SCI population. It should also be noted that as only peripheral nerve conduction was examined it is not possible to form any conclusions regarding potential central effects. Future research will be necessary to confirm or deny a lack of effect on central somatic nerve function. Finally, as the SNAP's recorded in the current study are representative of A-β, A-δ, and C fibers, it is not possible to make any conclusions regarding specific sensory fiber types. Future studies which utilize stimulations of varying frequencies will be necessary to differentiate between such fibers.

Conclusion

The present study demonstrated a lack of change in peripheral somatic nerve function despite the reduction of proinflammatory mediators in a population with SCI. Such results may suggest that, when at physiologically relevant concentrations, inflammatory mediators do not have a substantial enough influence to induce meaningful alterations in peripheral somatic nerve conduction. This result is of clinical significance, as it may suggest that despite previously reported ex-vivo findings, inflammatory mediators may not provide a suitable target for the enhancement of peripheral somatic nerve conduction in human populations with chronic inflammation.

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Conflict of Interest:

The authors declare no conflict of interest.

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Chapter 10

General Discussion

Traditionally, the study of the human body involves its categorization into various individual components such as the immune, endocrine, or nervous system and it is common practice for researchers and health care professionals to specialize in one particular system. This inherent way by which we study the body influences the way we characterize disease states and ultimately molds the way in which we treat different diseases. When an individual is found to demonstrate characteristics which deviate from what is considered 'normal' they are diagnosed with a disorder or disease which is typically characterized as belonging to one particular system. This characterization, then dictates the way in which treatment interventions are implemented. For example, as depression is characterized as a behavioural disorder, known to relate to abnormalities of neurotransmitters within the brain, the vast majority of treatment strategies involve pharmaceuticals which target such neurotransmitters. The major flaw concerning this traditional mode of characterizing and treating disease, is that, in reality, the body does not act as a collection of isolated systems, but rather as a single entity whereby individual 'systems' as we define them, work together as one and are able to communicate, interact, and influence one another. Based on this understanding it would seem inappropriate to characterize most disorders or diseases as belonging to one isolated system. Although a disorder may display more prominently in one system, it will almost certainly influence and in-turn be influenced by, a multitude of systems.

As such, a narrow focused or one-tiered approach to disease prevention and treatment may not provide maximum efficacy. Regardless of the traditional characterization of a disease or disorder, it is critical to gain an understanding of the potential influence and contribution of other systems. The use of an interdisciplinary

approach to the prevention or treatment of disease may lead to a better understanding of the complex nature of different pathologies, allow for the identification of the various systems involved, and further the establishment of novel and more efficacious treatment strategies.

The current prevalence of immune dysfunction in North America makes an understanding of the potential influence of the immune system on various aspects of health particularly important. Numerous metabolic conditions such as obesity, cardiovascular disease, and type 2 diabetes are now characterized as chronic inflammatory conditions, and as these conditions grow ever more prevalent, so too does the prevalence of chronic inflammation. The ability of the immune system to interact with other systems of the body by means of various mechanisms, makes it possible, and highly likely, that dysfunction within the immune system may spread and contribute to dysfunction in other systems. For example, the ability of various pro-inflammatory mediators to influence enzyme function provides a link between the immune system and the nervous system via the regulation of critical neuroactive compounds. Such compounds may act within the periphery, inducing effects such as nociceptor sensitization, or within the brain to induce behavioural deficits such as depression and cognitive impairment. Inflammatory mediators may also act in a more direct fashion and contribute to neural dysfunction, independent of their influence on neuroactive compounds.

Spinal cord injury is a condition which may be at a particularly high risk for inflammatory-induced neural and behavioural deficits. This is due in part to a susceptibility to immune dysfunction in the form of chronic inflammation stemming from

the high risk for the development of numerous metabolic conditions as well as the contraction of several common secondary health complications such UTI, or pressure ulcers. This population also demonstrates a much higher prevalence for numerous neural and behavioural disorders including depression, cognitive impairment, neuropathic pain, and somatic nerve deficits related to both motor and sensory impairment. As each of the disorders are notoriously difficult to treat, it was of value to examine novel intervention strategies which go beyond traditional treatment interventions and consider the potential contribution of the immune system. Such an approach has the potential to aid in the development of new treatment alternatives which may provide a more efficacious, and safe long-term strategy, devoid of the many side effects associated with traditional pharmaceutical treatment modalities.

Summary of Findings

Results from chapter 6 demonstrated that it was possible to improve mood and treat symptoms of depression in individuals with SCI by targeting the immune system and reducing levels of chronic inflammation. This was the first study to investigate the relationship between inflammation, its influence on neuroactive compounds of the kynurenine pathway, and symptoms of depression in individuals with SCI. These results suggest that the immune system may provide a viable target for intervention in individuals demonstrating chronic inflammation and symptoms of depression. The 55.1% improvement in CES-D scores obtained in the current study is comparable to that achieved in studies utilizing traditional pharmacological treatment strategies. In a study by Stahl 2000, 323 individuals with moderate to severe depression were treated with the SSRI's Citalopram or Sertraline over a 24-week period. The Citalopram group

experienced a 54.7% improvement and the Sertraline group experienced a 48.8% improvement based on scores from the Hamilton Depression Rating Scale (HAMD). Of these groups, adverse events resulted in a 14% and 19% dropout rate in the Citalopram and Sertraline groups respectively[1]. The improvement achieved in the current study was therefore comparable in magnitude to these SSRI's and acted in a shorter time frame while lacking negative side effects. Results from chapter 7 demonstrated a lack in response in most measures of cognitive function associated with verbal learning and memory despite a significant reduction in proinflammatory mediators. A significant improvement in intrusions (recall of incorrect off-list words) was the only aspect of the test to reach statistical significance. This may relate to the role of the frontal lobe in source memory and the ability to avoid incorrect responses[2]. While the hippocampus may have had preexisting hippocampal atrophy brought on by prolonged periods of chronic inflammation, the frontal lobe may not have been influenced as severely. Other mechanisms, more responsive to the intervention may have been responsible frontal lobe dysfunction. For example, individuals with cardiovascular disease (a chronic inflammatory condition) have been shown to demonstrate frontal lobe dysfunction which was correlated with cerebral blood flow and levels of CRP[3]. It is possible to speculate that the reduction in inflammation achieved in the current study was sufficient to improve cerebral blood flow, thereby improving frontal lobe function but not hippocampal function.

Results from chapter 8 demonstrated that it was possible to improve symptoms of neuropathic pain in individuals with SCI by targeting the immune system and reducing levels of chronic inflammation. This was the first study to examine the detailed

relationship between inflammatory mediators, corresponding concentrations of eicosanoids, and indices of neuropathic pain. The results suggest that the immune system may provide a suitable target for intervention in individuals with chronic inflammation and neuropathic pain following SCI. The significant 39.6% reduction in sensory neuropathic pain scores as assessed by the NPQ in the current study was comparable to that achieved by studies utilizing pharmacological treatment interventions. Gabapentin is a commonly used pharmaceutical for the treatment of neuropathic pain4. Studies have shown 8 weeks of Gabapentin administration to be sufficient to cause a 40.6% reduction in neuropathic pain scores in individuals with painful diabetic neuropathy[5], a 33.3% reduction in individuals with postherpetic neuralgia[6], and a 21.1% reduction in individuals with mixed neuropathic pain syndromes[7] based on an 11-point Likert scale. Significant changes occurred in these studies between 1-2 weeks following the initiation of treatment. According to Farrar et al. 2001, a 30% reduction in a pain intensity numerical rating scale (PI-NRS) represents a clinically important difference in pain[8]. Based on these results, the improvement in neuropathic pain scores achieved in the current study would be considered a clinically important difference and one that is comparable to that achieved following administration of gabapentin in painful diabetic neuropathy, and more effective than gabapentin administered to patients with postherpetic neuralgia or mixed neuropathic pain syndromes.

Results from chapter 9 demonstrated a lack of response in somatic nerve conduction, including both motor and sensory nerve conduction velocity and amplitude, despite a significant reduction in pro-inflammatory mediators. This was the first study to examine the influence of chronically elevated inflammatory mediators in humans, in-

vivo. These results may suggest that, despite previous findings in ex-vivo animal models, in humans, under physiologically relevant concentrations, inflammatory mediators do not seem to have a substantial influence on somatic nerve function.

Limitations and Future Directions

The results of the studies presented in this dissertation suggest that chronic inflammation may contribute to symptoms of depression and neuropathic pain and may therefore provide an appropriate target for intervention. Although these results are encouraging, several potential limitations should be noted. First, although the current sample size is considered relatively large in the area of SCI research, future, larger scale studies will be necessary in order to confirm such results and to further elucidate potential mechanisms. Physiological studies are frequently underpowered due to smaller sample sizes which may result in effects of practical importance going undetected. This may have been the case in the current study concerning changes in neuroactive compounds as many that were not significantly altered showed large effect sizes.

Second, due to the nature of the intervention in the current study it was not possible to blind participants to their condition. Although placebo supplements could have been provided to the control group, it was not possible to adequately blind participants to all aspects of the diet. This is a common issue/feature in clinical nutrition/diet intervention studies. The treatment group underwent a highly restrictive diet while the control group was free to consume their usual diet (which often consisted of unhealthy foods and foods from the ‘foods to avoid’ list supplied to the treatment group), making the differences in dietary intake between groups quite apparent. It may be possible for future studies to also alter the diet of the control group, in a way that will not

reduce inflammation, however, due to the easy access of nutritional information online, it will likely prove difficult to keep participants unaware of their group allocation. Alternatively, it may be possible for future studies to attempt to reduce inflammation by utilizing supplementation only, thereby allowing for the control group to unknowingly receive a placebo.

Third, it is not possible to elucidate which specific aspects of the dietary intervention induced the strongest anti-inflammatory effects. Although this was not the purpose of the present study, it will be necessary for future studies to examine various groups, utilizing various aspects of the diet along with a detailed mechanistic analysis of aspects such as transcription factor activity and membrane compositions in order to truly elucidate which features of the diet had the most substantial influence and by what means. Further, participants were allowed to continue exercising throughout the duration of the study and improvements in metabolic health induced by both the diet and exercise may have contributed to changes in inflammation. For example, although not assessed in the current study, reductions in adipose tissue may have contributed to the reductions in inflammation. Adipose tissue acts as an endocrine organ and possesses the ability to secrete proinflammatory mediators termed adipokines such as TNF-α, IL-1, and IL-6[9]. Therefore, a reduction in adipose tissue may have contributed to the reduction in such mediators. In addition, such adipokines have been suggested to play a role in insulin resistance via the downregulation of GLUT 4 gene transcription and translocation as well as the inhibition of insulin receptors by mechanisms related to the upregulation of the suppressors of cytokine signaling proteins[10,11]. Reductions in adipose tissue and adipokines may therefore also result in reduced blood glucose concentrations thereby

reducing strain on the kidneys and potential tissue damage which could contribute to further inflammation. Finally, improved cardiovascular health in the form of reduced platlet aggregation and plaque formation may contribute to reduced endothelial damage and related reductions in inflammation[12].

It may also prove extremely valuable for future studies to examine the efficacy of anti-inflammatory intervention strategies early in life. As prolonged periods of chronic inflammation may lead to potentially irreversible hippocampal atrophy, it may explain, in part, the difficulty in treating behavioural disorders such as depression and cognitive impairment later in life. Strategies aimed at reducing chronic inflammation in childhood may therefore help avoid the development of such intractable neuroanatomical complications and produce even more favorable results when treating behavioural disorders.

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Appendix

1. Raw Data

A) Blood Work

SUBJECT	CRP (ng/ml)			IL-2 (pg/ml)			IL-6 (pg/ml)			IL-1B (pg/ml)		
TREATMENT GROUP	*Baseline*	*Mid*	*Post*	*Baseline*	*Mid*	*Post*	*Baseline*	*Mid*	*Post*	*Baseline*	*Mid*	*Post*
S1	7733.64	4803.12	1659.10	6.23	2.37	0.85	3.12	1.12	0.29	3.89	1.15	0.51
S6	8559.22	11489.16	6542.75	0.19	0.07	0.12	1.48	0.17	0.66	0.19	0.02	0.18
S7	267.24	698.75	392.08	0.13	0.10	0.07	1.37	0.95	1.07	0.27	0.15	0.23
S8	359.76	571.13	275.39	0.94	0.37	0.38	1.50	0.16	0.14	0.46	0.26	0.26
S9	971.80	235.51	171.15	0.01	0.01	0.01	0.01	0.13	0.53	0.67	0.26	0.46
S11	3338.07	2934.22	1354.16	0.72	1.01	0.93	1.98	1.23	3.41	0.15	0.13	0.09
S12	1537.42	2380.87	4201.31	0.05	0.07	0.06	0.12	2.04	1.01	0.44	0.16	0.19
S13	7515.21	273.64	294.80	167.50	144.50	137.00	81.41	73.56	55.55	1.95	0.60	0.26
S15	2482.93	4171.47	2005.69	0.38	0.28	0.35	2.58	1.32	1.95	0.22	0.11	0.12
S16	3029.91	1835.54	3794.26	0.12	0.20	0.06	0.12	0.11	0.09	0.03	0.02	0.03
S17	9788.39	6981.35	6469.85	0.67	1.51	1.08	7.82	5.77	4.01	0.19	0.14	0.11
S18	8112.64	9496.30	7219.68	78.83	30.11	65.33	65.96	23.88	45.29	1.69	0.22	0.53
AVERAGE	*4474.69*	*3822.59*	*2865.02*	*21.31*	*15.05*	*17.19*	*13.96*	*9.20*	*9.50*	*0.85*	*0.27*	*0.25*
Standard Deviation	*3578.96*	*3749.39*	*2684.85*	*51.22*	*41.65*	*42.10*	*28.17*	*21.34*	*19.28*	*1.14*	*0.32*	*0.17*
CONTROL GROUP	*Baseline*	*Mid*	*Post*	*Baseline*	*Mid*	*Post*	*Baseline*	*Mid*	*Post*	*Baseline*	*Mid*	*Post*
S2	553.41	970.57	527.18	0.06	0.04	0.03	0.04	0.06	0.01	0.18	0.16	0.10
S3	1993.97	3431.78	2787.77	0.13	3.47	2.38	26.55	46.50	62.75	0.09	0.14	0.14
S4	9522.51	8307.97	11361.01	0.72	0.94	1.16	2.06	2.28	2.05	0.29	0.26	0.29
S5	1442.54	794.72	843.45	9.84	9.84	9.92	5.43	5.15	6.13	0.18	0.18	0.23
S10	1630.50	2720.57	912.61	0.01	0.01	0.03	3.67	0.16	0.26	0.12	0.16	0.20
S14	1988.34	6999.65	1860.60	1.82	1.89	3.16	10.22	6.66	8.61	0.94	0.60	0.66
S19	846.49	743.97	576.96	0.74	6.57	1.97	23.73	49.46	27.62	0.19	1.52	0.60
S20	1127.02	622.75	800.55	0.01	0.01	0.01	0.27	0.45	0.43	0.06	0.06	0.07
AVERAGE	*2388.10*	*3074.00*	*2458.77*	*1.67*	*2.85*	*2.33*	*9.00*	*13.84*	*13.48*	*0.26*	*0.39*	*0.29*
Standard Deviation	2928.09	3026.44	3678.85	3.36	3.62	3.29	10.50	21.22	21.92	0.29	0.49	0.22

TNF-alpha (pg/ml)			IFN-γ (pg/ml)			IL-4 (pg/ml)			IL-10 (pg/ml)			IL-1RA (pg/ml)		
Baseline	*Mid*	*Post*	*Baseline*	*Mid*	*Post*	*Baseline*	*Mid*	*Post*	*Baseline*	*Mid*	*Post*	*Baseline*	*Mid*	*Post*
19.24	19.87	7.81	30.60	22.73	13.73	0.00	0.00	0.00	5.26	0.74	0.66	43.75	33.19	25.17
9.38	1.15	6.72	5.81	0.03	0.90	0.00	19.07	0.00	0.13	0.00	0.05	10.56	2.40	8.77
12.00	15.25	14.42	2.79	3.38	1.61	0.00	0.00	0.00	0.00	0.00	0.00	4.05	5.03	5.37
7.86	8.61	7.19	23.96	2.80	2.94	0.00	0.00	0.00	0.84	1.44	3.43	57.68	29.82	30.85
8.44	10.99	12.02	8.52	10.57	22.40	15.86	54.65	112.50	0.00	0.00	0.00	19.38	40.07	53.49
13.55	13.73	11.94	2.28	1.79	1.57	0.00	0.00	0.00	0.35	1.82	0.97	29.60	21.07	25.99
15.38	15.56	14.09	0.68	0.76	0.60	0.00	0.00	0.00	0.00	0.09	0.00	0.92	1.30	1.42
12.13	10.65	11.07	267.50	180.00	153.67	0.00	0.00	0.00	2.53	0.84	0.15	50.38	23.32	20.08
8.89	6.98	7.03	21.21	13.26	9.61	0.00	0.00	0.00	1.45	2.07	0.94	93.50	54.69	34.96
11.78	8.96	9.88	0.41	0.11	0.20	0.00	0.00	0.00	0.00	0.19	0.04	17.68	57.82	39.98
17.64	20.29	20.87	34.77	27.09	8.80	2.33	5.97	0.03	36.65	103.00	73.16	30.95	36.42	23.88
13.76	9.99	11.48	236.67	121.00	204.00	72.10	68.68	81.85	31.08	24.66	31.54	38.16	28.91	46.09
12.50	*11.84*	*11.21*	*52.93*	*31.96*	*35.00*	*7.52*	*12.36*	*16.20*	*6.52*	*11.24*	*9.25*	*33.05*	*27.84*	*26.34*
3.62	*5.45*	*4.05*	*94.02*	*57.48*	*68.36*	*20.84*	*23.87*	*38.38*	*12.92*	*29.71*	*22.02*	*26.15*	*18.62*	*16.02*
Baseline	*Mid*	*Post*	*Baseline*	*Mid*	*Post*	*Baseline*	*Mid*	*Post*	*Baseline*	*Mid*	*Post*	*Baseline*	*Mid*	*Post*
9.01	7.51	8.94	0.64	0.46	0.21	0.00	0.00	0.00	0.00	0.00	0.00	22.03	11.56	9.04
16.18	26.79	37.35	139.00	231.50	280.00	0.00	0.00	0.00	0.00	0.01	0.00	207.33	302.00	321.50
6.88	6.92	8.43	12.18	11.70	22.40	0.00	0.00	0.00	5.11	4.15	7.46	17.95	17.49	20.67
10.12	9.77	8.63	23.38	13.57	15.10	68.05	61.23	68.91	41.21	39.36	41.89	89.91	88.43	88.36
15.29	13.70	16.26	1.68	0.81	4.81	0.00	0.00	0.00	0.09	0.00	0.00	15.85	13.88	8.81
7.40	8.68	8.59	7.85	8.01	9.62	0.00	0.00	0.00	0.00	0.00	0.00	73.97	91.83	105.00
8.10	11.17	9.06	38.99	123.00	62.83	90.42	238.00	121.50	0.39	1.66	1.21	53.15	86.94	72.15
5.44	5.85	6.54	0.84	1.49	1.63	0.00	0.00	0.00	0.05	0.09	0.04	4.61	5.28	4.84
9.80	*11.30*	*12.98*	*28.07*	*48.82*	*49.58*	*19.81*	*37.40*	*23.80*	*5.86*	*5.66*	*6.33*	*60.60*	*77.18*	*78.80*
3.92	6.74	10.26	46.75	84.55	95.27	37.16	83.84	46.26	14.39	13.70	14.60	66.62	98.58	105.79

PGE2 (pg/ml)			LTB4 (pg/ml)			TRP (ug/ml)			KYN (ng/ml)			KYN/TRP		
Baseline	*Mid*	*Post*	*Baseline*	*Mid*	*Post*	*Baseline*	*Mid*	*Post*	*Baseline*	*Mid*	*Post*	*Baseline*	*Mid*	*Post*
1481.12	1100.39	629.28	644.01	78.87	106.84	14.60	12.83	12.83	814.42	437.95	437.95	54.72	33.49	33.49
650.43	938.18	441.45	54.17	35.21	24.05	18.02	21.11	23.91	448.84	477.28	495.23	24.44	22.17	20.31
0.00	73.82	0.00	8.87	38.88	18.29	17.34	17.04	16.19	662.96	648.04	570.29	37.51	37.30	34.55
795.90	1991.25	1157.98	31.80	320.53	73.59	14.01	6.53	25.86	239.50	306.50	324.47	16.77	46.02	12.31
0.00	425.18	259.03	14.07	6.03	27.48	20.27	16.71	14.64	402.18	302.69	344.54	19.46	17.77	23.09
502.57	559.82	599.16	44.28	30.50	48.80	18.71	19.33	12.79	317.11	430.40	364.79	16.63	21.84	27.98
341.91	508.37	514.15	27.05	46.17	36.47	24.62	17.79	29.98	492.87	495.11	522.79	19.63	27.30	17.10
768.12	458.45	514.15	212.40	68.61	131.81	14.55	23.89	16.46	389.63	491.76	380.89	26.27	20.19	22.70
253.67	384.07	121.25	114.80	52.69	68.34	18.49	13.11	18.27	462.65	628.03	473.22	24.55	46.98	25.41
943.05	943.05	0.00	270.55	681.81	285.39	14.95	17.52	20.33	574.03	613.65	412.32	37.66	34.35	19.89
0.00	0.00	0.00	67.18	50.62	49.24	16.59	18.69	14.51	524.03	360.55	442.98	30.98	18.92	29.95
220.89	253.67	0.00	44.72	27.93	54.10	26.49	30.16	16.42	427.44	461.44	357.26	15.83	15.01	21.34
496.47	*636.35*	*353.04*	*127.82*	*119.82*	*77.03*	*18.22*	*17.89*	*18.51*	*479.64*	*471.12*	*427.23*	*27.04*	*28.45*	*24.01*
452.71	*544.91*	*357.47*	*181.81*	*194.99*	*73.76*	*3.96*	*5.87*	*5.46*	*153.41*	*115.92*	*77.03*	*11.56*	*10.99*	*6.60*
Baseline	*Mid*	*Post*	*Baseline*	*Mid*	*Post*	*Baseline*	*Mid*	*Post*	*Baseline*	*Mid*	*Post*	*Baseline*	*Mid*	*Post*
566.90	629.28	416.99	85.60	52.32	80.09	19.05	18.37	19.84	302.71	358.99	469.13	15.59	19.17	23.20
344.64	235.84	407.26	107.42	152.23	280.20	18.37	16.82	23.09	379.86	444.45	537.30	20.29	25.91	22.83
278.20	198.25	127.33	543.00	193.85	544.65	21.62	22.34	20.09	320.48	467.98	381.73	14.54	20.55	18.63
1442.98	1346.77	1361.30	57.09	43.99	54.79	19.71	28.93	20.89	404.89	458.87	489.17	20.15	15.56	22.97
355.27	292.94	571.11	57.17	28.77	50.98	18.99	21.13	16.66	496.23	498.48	490.65	25.63	23.14	28.89
629.81	485.07	860.33	20.97	85.43	53.92	21.77	18.05	18.16	356.56	480.76	445.23	16.06	26.12	24.05
1222.71	1427.76	1408.25	45.62	103.86	41.39	20.83	21.33	23.21	251.76	406.77	324.09	11.85	18.70	13.69
0.00	229.20	140.94	53.63	40.08	55.04	35.86	26.04	25.69	381.94	342.13	270.97	10.45	12.88	10.34
605.06	*605.64*	*661.69*	*121.31*	*87.57*	*145.13*	*22.02*	*21.63*	*20.95*	*361.81*	*432.30*	*426.03*	*16.82*	*20.25*	*20.58*
491.18	504.62	503.67	172.34	59.28	179.81	5.73	4.14	2.94	73.67	57.34	91.94	4.98	4.70	6.03

BCAA (umol/L)			Tyrosine (umol/L)			Phenylalanin (umol/L)			LNAA [sum] (umol/L)			TRP/LNAA (umol/L)			PHE/TYR		
Baseline	*Mid*	*Post*	*Baseline*	*Mid*	*Post*	*Baseline*	*Mid*	*Post*	*Baseline*	*Mid*	*Post*	*Baseline*	*Mid*	*Post*	*Baseline*	*Mid*	*Post*
497.90	339.04	342.87	91.08	58.87	36.82	59.75	49.90	41.72	648.73	447.81	421.41	110.19	140.25	149.04	656.05	847.56	1132.93
652.07	508.97	547.30	47.86	57.02	50.36	57.92	61.10	55.62	757.86	627.09	653.28	116.40	164.87	179.23	1210.25	1071.56	1104.39
992.79	447.64	519.19	49.54	85.97	63.61	38.02	53.07	44.57	1080.36	586.68	627.37	78.57	142.23	126.35	767.42	617.28	700.68
1871.00	390.57	835.21	43.43	38.16	70.09	50.45	47.11	62.13	1964.88	475.84	967.43	34.91	67.22	130.86	1161.69	1234.76	886.33
511.33	373.04	469.40	35.34	31.10	18.63	30.50	20.92	17.27	577.17	425.06	505.30	171.97	192.47	141.85	863.18	672.63	926.93
318.62	663.90	387.32	38.27	41.28	46.84	28.13	32.18	36.49	385.02	737.35	470.64	237.93	128.36	133.06	735.23	779.62	779.10
530.07	580.92	579.58	34.75	36.33	45.39	30.44	26.41	32.62	595.26	643.65	657.59	202.54	135.31	223.23	876.04	726.89	718.81
224.04	468.51	136.61	27.27	48.75	26.06	22.45	26.77	22.99	273.76	544.03	185.66	260.23	215.03	434.12	822.96	549.20	882.20
417.65	1231.34	267.76	32.56	47.37	29.90	23.52	34.64	21.48	473.73	1313.35	319.13	191.09	48.88	280.31	722.30	731.33	718.37
526.50	1455.28	765.61	62.85	54.09	40.72	46.01	36.72	27.49	635.36	1546.10	833.82	115.23	55.50	119.39	732.11	678.87	675.02
1245.96	545.47	413.58	52.03	41.29	27.16	22.28	16.64	16.95	1320.27	603.41	457.69	61.53	151.68	155.18	428.16	403.05	624.06
614.99	811.05	351.19	63.20	65.54	42.88	29.50	35.69	24.25	707.69	912.28	418.32	183.29	161.87	192.21	466.73	544.61	565.52
700.24	*651.31*	*467.97*	*48.18*	*50.48*	*41.54*	*36.58*	*36.76*	*33.63*	*785.01*	*738.55*	*543.14*	*146.99*	*133.64*	*188.74*	*786.84*	*738.11*	*809.53*
462.37	*351.97*	*198.51*	*17.70*	*15.14*	*15.24*	*13.64*	*13.58*	*14.84*	*468.17*	*352.28*	*217.26*	*71.30*	*52.25*	*90.42*	*232.24*	*229.53*	*180.36*
Baseline	*Mid*	*Post*	*Baseline*	*Mid*	*Post*	*Baseline*	*Mid*	*Post*	*Baseline*	*Mid*	*Post*	*Baseline*	*Mid*	*Post*	*Baseline*	*Mid*	*Post*
447.64	569.45	778.99	70.83	65.86	89.20	59.63	61.23	64.76	578.11	696.53	932.95	161.31	129.10	104.11	841.91	929.62	726.04
505.14	500.45	579.67	50.30	61.90	76.81	47.96	47.67	63.90	603.40	610.03	720.38	149.03	135.05	156.91	953.42	770.15	831.92
502.58	427.20	738.96	70.30	54.65	61.57	61.35	53.34	61.74	634.24	535.18	862.26	166.94	204.40	114.11	872.66	976.04	1002.69
459.14	441.25	522.60	60.84	77.69	77.69	58.03	63.34	77.89	578.01	582.28	678.18	166.97	243.29	150.81	953.92	815.31	1002.63
464.94	489.03	414.08	33.93	30.62	41.42	31.03	25.13	33.01	529.89	544.77	488.51	175.47	189.95	166.97	914.40	820.76	797.05
590.29	364.12	316.83	38.51	42.28	36.21	31.44	36.80	30.62	660.25	443.20	383.66	161.47	199.46	231.78	816.44	870.49	845.43
227.02	317.33	438.53	64.52	37.91	58.27	18.70	16.72	19.59	310.25	371.95	516.39	328.78	280.80	220.10	289.90	441.07	336.27
401.10	669.05	484.87	86.07	58.46	56.09	28.42	22.52	17.96	515.59	750.04	558.92	340.55	170.03	225.09	330.14	385.16	320.25
449.73	*472.23*	*534.32*	*59.41*	*53.67*	*62.16*	*42.07*	*40.84*	*46.18*	*551.22*	*566.75*	*642.66*	*206.31*	*194.01*	*171.24*	*746.60*	*751.08*	*732.78*
105.50	112.08	159.16	17.55	15.71	18.29	16.64	18.15	23.38	108.72	123.78	190.34	79.63	51.24	49.80	274.06	219.12	267.20

B) CES-D Scores of Depression

SUBJECT	CSE-D Score		
TREATMENT GROUP	***Baseline***	***Mid***	***Post***
S1	39	11	16
S6	4	2	5
S7	26	18	16
S8	22	6	6
S9	11	2	1
S11	7	1	1
S12	2	0	2
S13	20	7	12
S15	11	14	5
S16	8	5	4
S17	8	4	3
S18	16	11	12
AVERAGE	***14.50***	***6.75***	***6.92***
Standard Deviation	***10.67***	***5.64***	***5.58***
CONTROL GROUP	***Baseline***	***Mid***	***Post***
S2	18	15	22
S3	3	0	4
S4	11	7	10
S5	37	29	41
S10	22	19	20
S14	4	4	2
S19	15	16	18
S20	1	0	0
AVERAGE	***13.88***	***11.25***	***14.63***
Standard Deviation	**12.01**	**10.25**	**13.62**

C) CVLT Scores

SUBJECT	List A Total Trials Score			List A, Trial 1 Free Recall			List A, Trial 5 Free Recall			List B Free Recall Score		
TREATMENT GROUP	***Baseline***	***Mid***	***Post***	***Baseline***	***Mid***	***Post***	***Baseline***	***Mid***	***Post***	***Baseline***	***Mid***	***Post***
S1	55	58	62	6	8	9	14	15	15	5	5	7
S6	42	47	57	7	6	9	11	10	11	6	7	4
S7	34	48	51	6	7	8	7	10	12	4	6	6
S8	50	54	61	6	8	9	11	14	13	6	5	6
S9	48	50	59	7	7	9	11	12	15	5	3	8
S11	34	49	54	4	8	11	8	13	10	5	5	6
S12	39	37	45	5	5	8	8	8	9	4	4	4
S13	51	74	68	8	11	8	13	16	16	7	5	3
S15	43	48	42	4	6	4	10	12	10	5	7	4
S16	44	50	54	7	7	7	9	12	13	6	5	4
S17	55	53	61	7	7	10	13	14	12	9	7	5
S18	43	46	52	7	6	8	10	10	12	5	6	4
AVERAGE	***44.83***	***51.17***	***55.50***	***6.17***	***7.17***	***8.33***	***10.42***	***12.17***	***12.33***	***5.58***	***5.42***	***5.08***
Standard Deviation	***7.17***	***8.80***	***7.40***	***1.27***	***1.53***	***1.72***	***2.19***	***2.37***	***2.19***	***1.38***	***1.24***	***1.51***
CONTROL GROUP	***Baseline***	***Mid***	***Post***	***Baseline***	***Mid***	***Post***	***Baseline***	***Mid***	***Post***	***Baseline***	***Mid***	***Post***
S2	49	55	60	5	8	9	13	14	12	5	7	6
S3	61	69	65	6	9	8	16	14	15	5	8	6
S4	23	25	26	3	2	3	6	7	5	1	3	4
S5	51	57	53	6	8	8	10	13	10	4	3	5
S10	41	47	44	7	6	5	8	11	11	5	5	4
S14	56	58	57	8	8	8	14	14	13	7	5	5
S19	63	61	73	8	8	9	15	10	16	6	8	7
S20	49	57	58	5	7	8	13	14	14	4	4	5
AVERAGE	***49.13***	***53.63***	***54.50***	***6.00***	***7.00***	***7.25***	***11.88***	***12.13***	***12.00***	***4.63***	***5.38***	***5.25***
Standard Deviation	***12.72***	***13.08***	***14.27***	***1.69***	***2.20***	***2.12***	***3.52***	***2.59***	***3.46***	***1.77***	***2.07***	***1.04***

Short Delay Free Recall Score			Short Delay Cued Recall Score			Long Delay Free Recall Score			Long Delay Cued Recall Score			Long Delay Recognition Hits		
Baseline	*Mid*	*Post*	*Baseline*	*Mid*	*Post*	*Baseline*	*Mid*	*Post*	*Baseline*	*Mid*	*Post*	*Baseline*	*Mid*	*Post*
11	14	14	14	15	15	13	15	14	12	15	16	48	47	48
10	10	11	11	12	16	10	10	14	12	11	14	42	43	43
7	6	4	8	9	9	7	7	10	9	9	10	45	40	43
13	13	1	12	14	16	12	14	13	12	15	15	43	43	46
11	11	13	11	13	13	14	13	14	13	13	15	47	48	48
5	8	11	10	8	12	8	8	9	10	8	10	38	41	43
6	6	8	7	8	10	5	7	7	8	9	10	45	44	47
11	16	16	14	16	16	14	16	16	14	16	16	47	48	48
9	8	7	9	11	11	9	9	8	10	11	10	44	42	44
6	10	13	9	13	13	7	14	14	11	13	15	41	38	46
12	13	14	11	12	15	12	13	13	13	12	14	43	47	47
7	11	10	8	9	11	7	8	10	6	10	10	41	44	45
9.00	***10.50***	***10.17***	***10.33***	***11.67***	***13.08***	***9.83***	***11.17***	***11.83***	***10.83***	***11.83***	***12.92***	***43.67***	***43.75***	***45.67***
2.70	***3.15***	***4.45***	***2.27***	***2.71***	***2.50***	***3.10***	***3.33***	***2.89***	***2.33***	***2.62***	***2.64***	***2.93***	***3.25***	***2.02***
Baseline	*Mid*	*Post*	*Baseline*	*Mid*	*Post*	*Baseline*	*Mid*	*Post*	*Baseline*	*Mid*	*Post*	*Baseline*	*Mid*	*Post*
9	12	15	9	13	13	10	13	14	10	11	15	44	36	46
14	14	15	14	14	16	15	15	15	16	14	15	45	41	42
4	6	6	6	9	7	5	7	4	3	8	7	37	41	36
10	10	7	10	12	10	9	9	9	11	12	13	45	47	45
6	9	8	7	9	11	9	9	10	10	11	11	42	40	42
11	14	13	10	14	14	10	14	14	11	14	14	46	46	46
15	13	16	16	16	16	15	13	16	16	16	16	48	48	48
12	12	15	12	14	16	12	13	15	13	14	16	46	47	45
10.13	***11.25***	***11.88***	***10.50***	***12.63***	***12.88***	***10.63***	***11.63***	***12.13***	***11.25***	***12.50***	***13.38***	***44.13***	***43.25***	***43.75***
3.76	***2.76***	***4.16***	***3.38***	***2.50***	***3.31***	***3.34***	***2.88***	***4.12***	***4.13***	***2.51***	***3.07***	***3.36***	***4.33***	***3.73***

Long Delay False Positives			Total Intrusions			Total Repetitions		
Baseline	***Mid***	***Post***	***Baseline***	***Mid***	***Post***	***Baseline***	***Mid***	***Post***
0	1	0	0	1	4	2	13	4
4	4	5	7	1	4	6	0	6
1	5	4	6	5	4	1	1	3
2	5	2	10	0	1	7	3	2
0	0	0	1	0	0	1	6	3
8	5	4	11	7	12	2	0	3
2	2	0	3	1	2	12	1	5
0	0	0	0	0	1	8	0	2
0	2	0	4	1	0	1	2	0
6	8	2	5	12	2	0	4	8
2	0	0	2	0	3	12	5	14
1	0	0	15	9	6	6	5	7
2.17	***2.67***	***1.42***	***5.33***	***3.08***	***3.25***	***4.83***	***3.33***	***4.75***
2.59	***2.67***	***1.93***	***4.72***	***4.14***	***3.31***	***4.30***	***3.73***	***3.70***
Baseline	***Mid***	***Post***	***Baseline***	***Mid***	***Post***	***Baseline***	***Mid***	***Post***
4	12	1	8	4	5	16	12	9
3	6	6	4	20	34	4	18	10
6	5	10	11	12	28	2	0	5
1	0	2	4	0	3	5	9	5
6	7	6	10	15	26	5	14	3
1	0	0	1	0	2	1	2	2
0	0	0	0	0	0	4	10	0
1	1	3	2	1	0	4	4	2
2.75	***3.88***	***3.50***	***5.00***	***6.50***	***12.25***	***5.13***	***8.63***	***4.50***
2.38	***4.39***	***3.55***	***4.17***	***8.00***	***14.41***	***4.61***	***6.21***	***3.51***

D) NPQ Scores

SUBJECT	Sensory Pain Score			Affective Pain Score			Sensitivity Pain Score		
TREATMENT GROUP	***Baseline***	***Mid***	***Post***	***Baseline***	***Mid***	***Post***	***Baseline***	***Mid***	***Post***
S1	67.50	50.83	50.83	85.63	50.63	50.63	82.50	41.67	41.67
S6	39.17	25.00	15.83	45.00	14.29	15.00	0.00	0.00	0.00
S7	52.27	31.82	14.55	16.67	18.33	6.67	38.00	28.00	24.00
S8	25.33	6.33	17.00	43.13	15.00	17.50	29.00	18.00	14.00
S9	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
S11	35.00	20.00	25.00	30.00	23.33	35.00	20.00	50.00	50.00
S12	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
S13	24.72	19.44	32.78	53.75	41.25	38.13	30.00	28.00	57.00
S15	49.09	49.55	38.18	32.50	46.25	27.50	28.33	20.00	23.33
S16	58.75	55.00	24.38	77.86	66.43	51.43	62.50	63.75	40.00
S17	41.43	22.86	19.29	31.25	16.25	12.50	31.67	21.67	21.67
S18	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
AVERAGE	*32.77*	*23.40*	*19.82*	*34.65*	*24.31*	*21.20*	*26.83*	*22.59*	*22.64*
Standard Deviation	*23.34*	*20.16*	*15.80*	*28.56*	*21.94*	*19.03*	*26.05*	*21.19*	*20.72*
CONTROL GROUP	***Baseline***	***Mid***	***Post***	***Baseline***	***Mid***	***Post***	***Baseline***	***Mid***	***Post***
S2	25.00	30.67	37.00	39.38	27.50	33.13	20.83	34.17	34.17
S3	46.56	54.38	57.81	61.67	68.33	64.17	81.00	77.00	65.00
S4	11.00	8.50	9.00	16.67	6.67	6.67	60.00	46.67	56.67
S5	34.67	54.00	28.67	57.14	30.00	57.14	62.50	55.00	60.00
S10	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
S14	23.75	36.25	26.25	35.00	23.33	18.33	10.00	40.00	30.00
S19	3.75	17.50	11.25	10.00	5.00	10.00	3.33	16.67	15.00
S20	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
AVERAGE	*18.09*	*25.16*	*21.25*	*27.48*	*20.10*	*23.68*	*29.71*	*33.69*	*32.60*
Standard Deviation	*17.22*	*22.17*	*20.06*	*24.43*	*23.01*	*25.28*	*32.85*	*26.99*	*26.25*

E) Somatic Nerve Conduction Scores

SUBJECT	Motor NCV			M-Wave Amplitude			Sensory NCV			Sensory Amplitude		
TREATMENT GROUP	***Baseline***	***Mid***	***Post***	***Baseline***	***Mid***	***Post***	***Baseline***	***Mid***	***Post***	***Baseline***	***Mid***	***Post***
S1	48	50.1	48	3.1	1.78	1.97	--	49.2	40.7	--	4.4	5.9
S6	53.3	50.8	52	4.4	5.4	4.9	45.6	41.6	35.5	5.5	4.9	3.8
S7	55.1	53.4	54.3	4.1	4.5	5.4	39.6	40.3	37.5	8.1	5.9	6.3
S8	47.6	46.8	51.4	5.4	7.7	5.2	31.3	35.1	32.7	5.3	5.5	4.5
S9	--	--	--	--	--	--	50.5	55.7	45.5	7.3	5.6	7.8
S11	55.1	46	51.6	4.3	4.4	2.4	47.3	35.5	40.5	5	3.6	3.5
S12	50.6	51.2	53.8	6.4	5.2	4.2	34.1	35.9	33.6	8	8.7	6.1
S13	49.5	53.2	56.6	1.28	0.69	0.48	43.2	48.7	43.1	14.4	9	4
S15	39.7	47.3	50	2.3	1.72	1.38	--	--	--	--	--	
S16	58.5	53.4	56.3	3	1.51	1.09	44.3	40.9	48.3	10.5	8.2	4.6
S17	35.7	40.4	36.8	4.9	6	4.8	36.1	38.6	36.4	9.8	8.9	6.3
S18	54.1	47	52.6	4.6	4.8	4.1	62.5	54.9	52.1	11.5	8.7	5.7
AVERAGE	***49.75***	***49.05***	***51.22***	***3.98***	***3.97***	***3.27***	***43.45***	***43.31***	***40.54***	***8.54***	***6.67***	***5.32***
Standard Deviation	***6.87***	***4.00***	***5.42***	***1.46***	***2.22***	***1.83***	***9.05***	***7.58***	***6.22***	***3.03***	***2.04***	***1.33***
CONTROL GROUP	***Baseline***	***Mid***	***Post***	***Baseline***	***Mid***	***Post***	***Baseline***	***Mid***	***Post***	***Baseline***	***Mid***	***Post***
S2	54.3	54.7	53.4	4.9	4.9	5.3	51.7	53.7	52.4	15	15.2	11.6
S3	52.4	56.2	48.2	4	2.5	1.86	35.5	37.1	45.9	8.8	4.9	3.7
S4	--	--	--	--	--	--	42.9	48.4	48.4	7.9	6.5	6.5
S5	43.8	46.7	42.5	6	5.7	5.8	37	37.7	26.9	14	10.1	6.7
S10	52	52.6	51.1	5.3	5.1	5.8	35.7	36.6	37.6	5.8	3.4	3.9
S14	45.7	50.1	58.2	6.1	7.7	7.5	44.8	49.8	47.6	12.8	13.9	11.5
S19	59.6	55.7	56.5	6	5.6	5.2	56.7	53.4	50.2	14.8	16.9	12
S20	56.2	50.2	59.9	9	9.4	9.5	48.6	50	50.5	7.8	6.5	5.4
AVERAGE	***52.00***	***52.31***	***52.83***	***5.90***	***5.84***	***5.85***	***44.11***	***45.84***	***44.94***	***10.86***	***9.68***	***7.66***
Standard Deviation	***5.59***	***3.49***	***6.11***	***1.56***	***2.19***	***2.33***	***7.87***	***7.43***	***8.57***	***3.67***	***5.11***	***3.51***

2. Inflammatory Mediators – Normative Values

Cytokine	Kim et al. 2011	Treatment Baseline	Control Baseline
IL-2	5.13pg/ml	21.3pg/ml	1.7pg/ml
IL-6	2.92pg/ml	13.9pg/ml	9.0pg/ml
TNF-alpha	3.21pg/ml	12.5pg/ml	9.8pg/ml
IFN-γ	13.1pg/ml	52.9pg/ml	28.1pg/ml
IL-10	1.32pg/ml	6.5pg/ml	5.9pg/ml
IL-4	0.6pg/ml	7.5pg/ml	19.8pg/ml
	Di Lorio et al. 2003		
IL-1B	0.3pg/ml	0.9pg/ml	0.3pg/ml
	Greenland et al. 2010		
CRP	<1000ng/ml	4474.7ng/ml	2388.1ng/ml
	Hurme et al. 1998		
IL-1RA	681pg/ml	33.1pg/ml	60.6pg/ml

3. Center for Epidemiological Studies Depression Scale

Depression Screening

Center for Epidemiologic Studies Depression (CES-D)

Scale Description:
The following scale was developed by the Center for Epidemiologic Studies (Radlof, 1977). The scale has been found reliable (Alpha>.85) in previous research (Hann et. al., 1999). A Spanish version of this scale is also available.

Scale items:
Below is a list of some ways you may have felt or behaved. Please indicate how often you have felt this way during the last week by checking the appropriate space. Please only provide one answer to each question.

	During the past week:	*Rarely* or none of the time (less than 1 day)	*Some* or a little of the time (1-2 days)	*Occasionally* or a moderate amount of time (3-4 days)	*Most* or all of the time (5-7 days)
1.	I was bothered by things that usually don't bother me.				
2.	I did not feel like eating; my appetite was poor.				
3.	I felt that I could not shake off the blues even with help from my family or friends.				
4.	I felt I was just as good as other people.				
5.	I had trouble keeping my mind on what I was doing.				
6.	I felt depressed.				
7.	I felt that everything I did was an effort.				
8.	I felt hopeful about the future.				
9.	I thought my life had been a failure.				
10.	I felt fearful.				
11.	My sleep was restless.				
12.	I was happy.				
13.	I talked less than usual.				
14.	I felt lonely.				
15.	People were unfriendly.				
16.	I enjoyed life.				
17.	I had crying spells.				
18.	I felt sad.				
19.	I felt that people disliked me.				
20.	I could not get going.				

Scoring:	Rarely (Less than 1 day)	Some (1-2 days)	Occasionally (3-4 days)	Most (5-7 days)
Questions 4, 8, 12, and 16	3	2	1	0
All other questions	0	1	2	3

The score is the sum of the 20 questions. Possible range is 0-60. If more than four questions are missing answers, do not score the CES-D questionnaire. A score of 16 points or more is considered depressed.

4. Neuropathic Pain Questionnaire

Neuropathic Pain Questionnaire

In order to assess and treat your pain problem, we need to thoroughly understand just exactly what type of pain you have, and how it may or may not change over time. You may have only one site of pain, or you may have more than
one.

Please name the site of pain which is most severe or disturbing for you (eg, arm, foot, etc):
_____

For all of the following questions, please rate your pain at the site you just listed.
Please use the space below to describe your pain in your own words as well:

Please use the items below to rate your pain as it usually feels. Indicate a number which represents your pain on each scale. For example, if you have no tingling pain, you would rate the first item "0". If you have the worst tingling pain imaginable, you would rate it "100." If neither of those fits your pain because it is in between, choose a number which fits your pain.

Sensory Items

1. Burning Pain

   0 <==========>100 Please rate
   No burning Worst burning your usual pain:
   _____
   pain pain imaginable

2. Sharp Pain

   0 <==========>100 Please rate
   No sharp Worst sharp your usual pain:
   _____
   pain pain imaginable

3. Dull, Aching Pain

   0 <==========>100 Please rate
   No aching Worst aching your usual pain:
   _____
   pain pain imaginable

4. Overly Sensitive to Touch

   0 <==========>100 Please rate
   No increase Greatest increase your usual pain:
   _____
   at all imaginable

5. Shooting Pain

0 <==========>100 Please rate
No shooting Worst shooting your usual pain:
_____
pain pain imaginable

6. Throbbing Pain

0 <==========>100 Please rate
No throbbing Worst throbbing your usual pain:
_____
pain pain imaginable

7. Stabbing Pain

0 <==========>100 Please rate
No stabbing Worst stabbing your usual pain:
_____
pain pain imaginable

8. Numbness

0 <==========>100 Please rate
No numbness Worst numbness your usual pain:
_____
imaginable

9. Electric Pain

0 <==========>100 Please rate
No electric Worst electric your usual pain:
_____
pain pain imaginable

10. Grinding Pain

0 <==========>100 Please rate
No grinding Worst grinding your usual pain:
_____
pain pain imaginable

11. Tearing Pain

0 <==========>100 Please rate
No tearing Worst tearing your usual pain:
_____
pain pain imaginable

12. Cramping Pain

0 <==========>100 Please rate

No cramping Worst cramping your usual pain: _____
pain pain imaginable

13. Soreness

0 <==========>100 Please rate
No soreness Worst soreness your usual pain: _____
imaginable

14. Tingling Pain

0 <==========>100 Please rate
No tingling Worst tingling your usual pain: _____
pain pain imaginable

15. Squeezing Pain

0 <==========>100 Please rate
No squeezing Worst squeezing your usual pain: _____
pain pain imaginable

16. Itching Pain

0 <==========>100 Please rate
No itching Worst itching your usual pain: _____
pain pain imaginable

17. Freezing Pain

0 <==========>100 Please rate
No freezing Worst freezing your usual pain: _____
pain pain imaginable

18. Pinching Pain

0 <==========>100 Please rate
No pinching Worst pinching your usual pain: _____
pain pain imaginable

We are also interested in learning how your pain effects you. Please write the number that indicates the amount you experience each of the following:

Affective Items

19. How unpleasant is your usual pain?

0 <==========>100 Please rate
Not unpleasant Most unpleasant your usual pain:
_____
at all pain imaginable

20. How annoying is your usual pain?

0 <==========>100 Please rate
Not annoying Most annoying your usual pain:
_____
at all pain imaginable

21. How frightening is your usual pain?

0 <==========>100 Please rate
Not frightening Most frightening your usual pain:
_____
at all pain imaginable

22. How confusing is your usual pain?

0 <==========>100 Please rate
Not confusing Most confusing your usual pain:
_____
at all pain imaginable

23. How distressing is your usual pain?

0 <==========>100 Please rate
Not distressing Most distressing your usual pain:
_____
at all pain imaginable

24. How irritating is your usual pain?

0 <==========>100 Please rate
Not irritating Most irritating your usual pain:
_____
at all pain imaginable

25. How overwhelming is your usual pain?

0 <==========>100 Please rate
Not overwhelming Most overwhelming your usual pain:
_____
at all pain imaginable

26. How exasperating is your usual pain?

0 <==========>100 Please rate
Not exasperating Most exasperating your usual pain:
_____
at all pain imaginable

We are also interested in learning what circumstances cause changes in your pain. Please write the number that indicates the amount you experience each of the following:

Sensitivity Items

27. Increased pain due to heat

0 <==========>100 Please rate
No increase Greatest increase your usual pain:
_____
at all imaginable

28. Increased pain due to cold

0 <==========>100 Please rate
No increase Greatest increase your usual pain:
_____
at all imaginable

29. Increased pain due to touch

0 <==========>100 Please rate
No increase Greatest increase your usual pain:
_____
at all imaginable

30. Increased pain to fatigue

0 <==========>100 Please rate
No increase Greatest increase your usual pain:
_____
at all imaginable

31. Increased pain due to emotional upset

0 <==========>100 Please rate
No increase Greatest increase your usual pain:
_____
at all imaginable

32. Increased pain due to weather changes

0 <==========>100 Please rate
No increase Greatest increase your usual pain:
_____
at all imaginable

5. Ethical Approval

Brock University
Research Ethics Office

Tel: 905-688-5550 ext. 3035
Email: reb@brocku.ca

Bioscience Research Ethics Board

Certificate of Ethics Clearance for Human Participant Research

DATE: 9/23/2014

PRINCIPAL INVESTIGATOR: DITOR, David - Kinesiology

FILE: 13-192 - DITOR

TYPE: Ph. D. STUDENT: David Allison
SUPERVISOR: David Ditor

TITLE: Neural Consequences of Chronic Inflammation in Spinal Cord Injury

ETHICS CLEARANCE GRANTED

Type of Clearance: NEW Expiry Date: 9/30/2015

The Brock University Bioscience Research Ethics Board has reviewed the above named research proposal and considers the procedures, as described by the applicant, to conform to the University's ethical standards and the Tri-Council Policy Statement. Clearance granted from **9/23/2014** to **9/30/2015**.

The Tri-Council Policy Statement requires that ongoing research be monitored by, at a minimum, an annual report. Should your project extend beyond the expiry date, you are required to submit a Renewal form before 9/30/2015. Continued clearance is contingent on timely submission of reports.

To comply with the Tri-Council Policy Statement, you must also submit a final report upon completion of your project. All report forms can be found on the Research Ethics web page at http://www.brocku.ca/research/policies-and-forms/research-forms.

In addition, throughout your research, you must report promptly to the REB:

a) Changes increasing the risk to the participant(s) and/or affecting significantly the conduct of the study;
b) All adverse and/or unanticipated experiences or events that may have real or potential unfavourable implications for participants;
c) New information that may adversely affect the safety of the participants or the conduct of the study;
d) Any changes in your source of funding or new funding to a previously unfunded project.

We wish you success with your research.

Approved: 

_____

Brian Roy, Chair
Bioscience Research Ethics Board

Note: Brock University is accountable for the research carried out in its own jurisdiction or under its auspices and may refuse certain research even though the REB has found it ethically acceptable.

If research participants are in the care of a health facility, at a school, or other institution or community organization, it is the responsibility of the Principal Investigator to ensure that the ethical guidelines and clearance of those facilities or institutions are obtained and filed with the REB prior to the initiation of research at that site.

6. Review Papers

Allison DJ & Ditor DS. The common inflammatory etiology of depression and cognitive impairment: A therapeutic target. Journal of Neuroinflammation, September 2014, 11(1):151.

Allison and Ditor *Journal of Neuroinflammation* 2014, **11**:151
http://www.jneuroinflammation.com/content/11/1/151

JOURNAL OF NEUROINFLAMMATION

REVIEW Open Access

The common inflammatory etiology of depression and cognitive impairment: a therapeutic target

David J Allison* and David S Ditor

Abstract

Chronic inflammation has been shown to contribute to the development of a wide variety of disorders by means of a number of proposed mechanisms. Depression and cognitive impairment are two such disorders which may share a closely linked inflammatory etiology. The ability of inflammatory mediators to alter the activity of enzymes, from key metabolic pathways, may help explain the connection between these disorders. The chronic up-regulation of the kynurenine pathway results in an imbalance in critical neuroactive compounds involving the reduction of tryptophan and elevation of tryptophan metabolites. Such imbalances have established implications in both depression and cognitive impairment. This may implicate the immune system as a potential therapeutic target in the treatment of these disorders. The most common treatment modalities currently utilized, involve drug interventions which act on downstream targets. Such treatments help to reestablish protein balances, but fail to treat the inflammatory basis of the disorder. The use of anti-inflammatory interventions, such as regular exercise, may therefore, contribute to the effectiveness of current drug interventions in the treatment of both depression and cognitive impairment.

Keywords: Indoleamine 2,3-dioxygenase, Tryptophan 2,3-dioxygenase, Kynurenine pathway, Chronic Inflammation, Depression, Cognitive impairment, Exercise

Background

Chronic inflammation plays an increasingly appreciated role in the pathogenesis of a number of neurological and behavioral disorders including depression and cognitive impairment [1-3]. In addition, chronic inflammation contributes to the pathogenesis of a number of related metabolic disorders, and these disorders in turn have been shown to contribute to the elevated inflammatory state, creating a vicious cycle [4-8]. Consequently, as conditions such as obesity, cardiovascular disease, and diabetes become ever more prevalent, so too does the occurrence of chronic inflammation. Communicatory pathways between systems allow for immune dysfunction to contribute to both neural and endocrinal impairment via a number of inflammatory mechanisms. Proinflammatory mediators possess the ability to directly influence the nervous system by acting on vagal afferents [9], or by crossing the blood brain barrier (BBB) either through leaky sites at the circumventricular organs [10], or via specialized active transporters [11]. Proinflammatory cytokines have also been shown to influence hormone secretion by acting directly on receptors within the hypothalamic-pituitary-adrenal (HPA) axis [12]. Alternatively, a number of cytokines have been shown to indirectly influence neural and endocrinal disorders by altering the regulation of enzymes. This may result in a shift in key metabolic pathways resulting in an imbalance in critical neuroactive compounds.

Both depression and cognitive impairment may share a closely linked inflammatory etiology stemming from a cytokine-induced imbalance in the kynurenine pathway. As this pathway provides the primary route for tryptophan (TRP) degradation, it plays a major role not only in the maintenance of serotonin (5-HT) synthesis, but also in the critical balance between neurotoxic and neuroprotective metabolites. As such, a state of chronic inflammation, as is commonly reported in cases of depression and severe cognitive deficits, may contribute to the pathogenesis of each of these disorders [13-16].

Consequently, the kynurenine pathway has become a prospective target for treatment interventions. However, the majority of current intervention strategies for depression and cognitive impairment utilize drugs which target

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Allison DJ & Ditor DS. Immune dysfunction and chronic inflammation following spinal cord injury. Spinal Cord, September 2014, 53:14-18.

Spinal Cord (2014), 1–5

www.nature.com/sc

REVIEW

Immune dysfunction and chronic inflammation following spinal cord injury

DJ Allison and DS Ditor

Study design: Review article.
Objectives: The objective of this study is to provide an overview of the many factors that contribute to the chronic inflammatory state typically observed following spinal cord injury (SCI).
Methods: Literature review.
Results: Not applicable.
Conclusion: SCI is typically characterized by a low-grade inflammatory state due to a number of factors. As bidirectional communication exists between the nervous, endocrine and immune systems, damage to the spinal cord may translate into both endocrinal and immune impairment. Damage to the autonomic nervous system may induce immune dysfunction directly, through the loss of neural innervation of lymphoid organs, or indirectly by inducing endocrinal impairment. In addition, damage to the somatic nervous system and the corresponding loss of motor and sensory function increases the likelihood of developing a number of secondary health complications and metabolic disorders associated with a state of inflammation. Lastly, numerous related disorders associated with a state of chronic inflammation have been found to be at a substantially higher prevalence following SCI. Together, such factors help explain the chronic inflammatory state and immune impairment typically observed following SCI. An understanding of the interactions between systems, both in health and disease, and the many causes of chronic inflammation may aid in the effective future treatment of immune dysfunction and related disorders following SCI.
Spinal Cord (2014) **00**, 1–5. doi:10.1038/sc.2014.184

INTRODUCTION

Spinal cord injury (SCI) is a condition commonly associated with immune impairment and a state of chronic inflammation. This has been demonstrated by elevated levels of circulating proinflammatory cytokines and autoantibodies, which are apparent in individuals who are symptomatic or asymptomatic for secondary health complications.[1–5] As such, this population is often found to be in a perpetual low-grade inflammatory state, which is elevated to an even further extent when other health complications and associated disorders are present.

Owing to the complex bidirectional communication between the immune and neuroendocrine systems, damage to the spinal cord often results in the widespread dysfunction in other systems.[6] Both the endocrine and immune system may be affected due to respective dysregulation of the hypothalamic-pituitary-adrenal (HPA) axis and a loss of direct sympathetic innervation of lymphoid organs. There is ample evidence demonstrating that despite an elevated inflammatory state, SCI is associated with a state of immunosuppression and a heightened susceptibility to infection.[6–9] Suppressed function of natural killer cells, neutrophils, macrophages and lymphocytes have each been documented following SCI.[6,10,11] The loss of motor and sensory function also contribute to this population's greater susceptibility to a number of acute infections, including urinary tract infection (UTI) and pressure ulcers, as well as metabolic disorders associated with a more sedentary lifestyle such as obesity, atherosclerosis and type 2 diabetes.[12–14] A number of related disorders found to be significantly more prevalent following SCI such as depression and neuropathic pain may also contribute to a heightened systemic inflammatory state.[15–17]

It has yet to be definitively established whether or not such elevations in proinflammatory mediators are beneficial to this population or if they are in fact surrogate markers of further neurological and endocrinal impairment. Such mediators have critical roles in the elimination of invading pathogens and the repair of damaged tissue; however, there is also evidence to suggest a number of pathological roles. As such, an understanding of the bidirectional influence concerning the ability of inflammatory mediators to both influence, and be influenced by, a variety of disorders and secondary health complications following SCI may aid in future treatment strategies.

Rationale for immune impairment and chronic inflammation following SCI

Autonomic nerve damage. The immune system is under neuromodulatory control via the direct innervation of primary and secondary lymphoid tissues by autonomic nerve fibers of the sympathetic nervous system.[10] Preganglionic sympathetic neurons originating from the spinal column synapse in the ganglia with peripheral sympathetic neurons. These nerve fibers are responsible for innervating several lymphoid organs and their blood vessels including the spleen, thymus and lymph nodes, whereby they act to regulate blood flow as well as

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